For example, the ketone is reduced by treatment with sodium borohydride in an alcoholic solvent such as isopropanol, at ambient temperature, to afford the alcohol **91.10**.

The alcohol **91.6** can be converted into the thiol **91.13** and the amine **91.14**, by means of displacement reactions with suitable nucleophiles, with inversion of stereochemistry. For example, the alcohol **91.6** can be converted into an activated ester such as the trifluoromethanesulfonyl ester or the methanesulfonate ester **91.7**, by treatment with methanesulfonyl chloride and a base. The mesylate **91.7** is then treated with a sulfur nucleophile, for example potassium thioacetate, as described in *Tetrahedron Lett.*, 1992, 4099, or sodium thiophosphate, as described in *Acta Chem. Scand.*, 1960, 1980, to effect displacement of the mesylate, followed by mild basic hydrolysis, for example by treatment with aqueous ammonia, to afford the thiol **91.13**.

For example, the mesylate **91.7** is reacted with one molar equivalent of sodium thioacetate in a polar aprotic solvent such as, for example, dimethylformamide, at ambient temperature, to afford the thioacetate **91.12**, in which R is $COCH_3$. The product then treated with, a mild base such as, for example, aqueous ammonia, in the presence of an organic co-solvent such as ethanol, at ambient temperature, to afford the thiol **91.13**.

The mesylate **91.7** can be treated with a nitrogen nucleophile, for example sodium phthalimide or sodium bis(trimethylsilyl)amide, as described in Comprehensive Organic Transformations, by R. C. Larock, p399, followed by deprotection as described previously, to afford the amine **91.14**.

For example, the mesylate **91.7** is reacted, as described in *Angew. Chem. Int. Ed.*, 7, 919, 1968, with one molar equivalent of potassium phthalimide, in a dipolar aprotic solvent, such as, for example, dimethylformamide, at ambient temperature, to afford the displacement product **91.8**, in which NR^aR^b is phthalimido. Removal of the phthalimido group, for example by treatment with an alcoholic solution of hydrazine at ambient temperature, as described in *J. Org. Chem.*, 38, 3034, 1973, then yields the amine **91.14**.

The application of the procedures described above for the conversion of the β-carbinol **91.6** to the α-thiol **91.13** and the α-amine **91.14** can also be applied to the α-carbinol **91.10**, so as to afford the β-thiol and β-amine, **91.11**.

Scheme **92** illustrates the preparation of compounds in which the phosphonate moiety is attached to the decahydroisoquinoline by means of a heteroatom and a carbon chain.

In this procedure, an alcohol, thiol or amine **92.1** is reacted with a bromoalkyl phosphonate **92.2**, under the conditions described above for the preparation of the phosphonate **90.4** (Scheme **90**), to afford the displacement product **92.3**. Removal of the ester group, followed by conversion of the acid to the R^4R^5N amide and N-deprotection, as described herein, (Scheme **96**) then yields the amine **92.8**.

For example, the compound **92.5**, in which the carboxylic acid group is protected as the trichloroethyl ester, as described in Protective Groups in Organic Synthesis, by T. W. Greene and P.G.M. Wuts, Wiley, 1991, p. 240, and the amine is protected as the cbz group, is reacted with a dialkyl 3-bromopropylphosphonate, **92.6**, the preparation of which is described in *J. Am. Chem. Soc.*, 2000, 122, 1554 to afford the displacement product **92.7**. Deprotection of the ester group, followed by conversion of the acid to the R^4R^5N amide and N-deprotection, as described herein, (Scheme **96**) then yields the amine **92.8**.

Using the above procedures, but employing, in place of the α-thiol **92.5**, the alcohols, thiols or amines **91.6, 91.10, 91.11, 91.13, 91.14**, of either α- or β-orientation, there are obtained the corresponding products **92.4**, in which the orientation of the side chain is the same as that of the O, N or S precursors.

Scheme **93** illustrates the preparation of phosphonates linked to the decahydroisoquinoline moiety by means of a nitrogen atom and a carbon chain. The compounds are prepared by means of a reductive amination procedure, for example as described in Comprehensive Organic Transformations, by R. C. Larock, p421.

In this procedure, the amines **91.14** or **91.11** are reacted with a phosphonate aldehyde **93.1**, in the presence of a reducing agent, to afford the alkylated amine **93.2**. Deprotection of the ester group, followed by conversion of the acid to the R^4NH amide and N-deprotection, as described herein, (Scheme **96**) then yields the amine **93.3**.

For example, the protected amino compound **91.14** is reacted with a dialkyl formylphosphonate **93.4**, the preparation of which is described in US Patent 3784590, in the presence of sodium cyanoborohydride, and a polar organic solvent such as ethanolic acetic acid, as described in *Org. Prep. Proc. Int.*, 11, 201, 1979, to give the amine phosphonate **93.5**. Deprotection of the ester group, followed by conversion of the acid to the R^4R^5N amide and N-deprotection, as described herein, (Scheme **96**) then yields the amine **93.6**.

Using the above procedures, but employing, instead of the α-amine **91.14**, the β isomer, **91.11** and/or different aldehydes **93.1**, there are obtained the corresponding products **93.3**, in which the orientation of the side chain is the same as that of the amine precursor.

Scheme **94** depicts the preparation of a decahydroisoquinoline phosphonate in which the phosphonate moiety is linked by means of a sulfur atom and a carbon chain.

In this procedure, a thiol phosphonate **94.2** is reacted with a mesylate **94.1**, to effect displacement of the mesylate group with inversion of stereochemistry, to afford the thioether product **94.3**. Deprotection of the ester group, followed by conversion of the acid to the R^4R^5N amide and N-deprotection, as described herein, (Scheme **96**) then yields the amine **94.4**.

For example, the protected mesylate **94.5** is reacted with an equimolar amount of a dialkyl 2-mercaptoethyl phosphonate **94.6**, the preparation of which is described in *Aust. J. Chem.*, 43, 1123, 1990. The reaction is conducted in a polar organic solvent such as ethanol, in the presence of a base such as, for example, potassium carbonate, at ambient temperature, to afford the thio ether phosphonate **94.7**. Deprotection of the ester group, followed by conversion of the acid to the R^4R^5N amide and N-deprotection, as described herein, (Scheme **96**) then yields the amine **94.8**

Using the above procedures, but employing, instead of the phosphonate **94.6**, different phosphonates **94.2**, there are obtained the corresponding products **94.4**.

Scheme **95** illustrates the preparation of decahydroisoquinoline phosphonates **95.4** in which the phosphonate group is linked by means of an aromatic or heteroaromatic ring. The compounds are prepared by means of a displacement reaction between hydroxy, thio or amino substituted substrates **95.1** and a bromomethyl substituted phosphonate **95.2**. The reaction is performed in an aprotic solvent in the presence of a base of suitable strength, depending on the nature of the reactant **95.1**. If X is S or NH, a weak organic or inorganic base such as triethylamine or potassium carbonate can be employed. If X is O, a strong base such as sodium hydride or lithium hexamethyldisilylazide is required. The displacement reaction affords the ether, thioether or amine compounds **95.3**. Deprotection of the ester group, followed by conversion of the acid to the R^4R^5N amide and N-deprotection, as described herein, (Scheme **96**) then yields the amine **95.4**.

For example, the protected alcohol **95.5** is reacted at ambient temperature with a dialkyl 3-bromomethyl phenylmethylphosphonate **95.6**, the preparation of which is described above,

(Scheme **80**). The reaction is conducted in a dipolar aprotic solvent such as, for example, dioxan or dimethylformamide. The solution of the carbinol is treated with one equivalent of a strong base, such as, for example, lithium hexamethyldisilylazide, and to the resultant mixture is added one molar equivalent of the bromomethyl phosphonate **95.6**, to afford the product **95.7**. Deprotection of the ester group, followed by conversion of the acid to the R^4R^5N amide and N-deprotection, as described herein, (Scheme **96**) then yields the amine **95.8**.

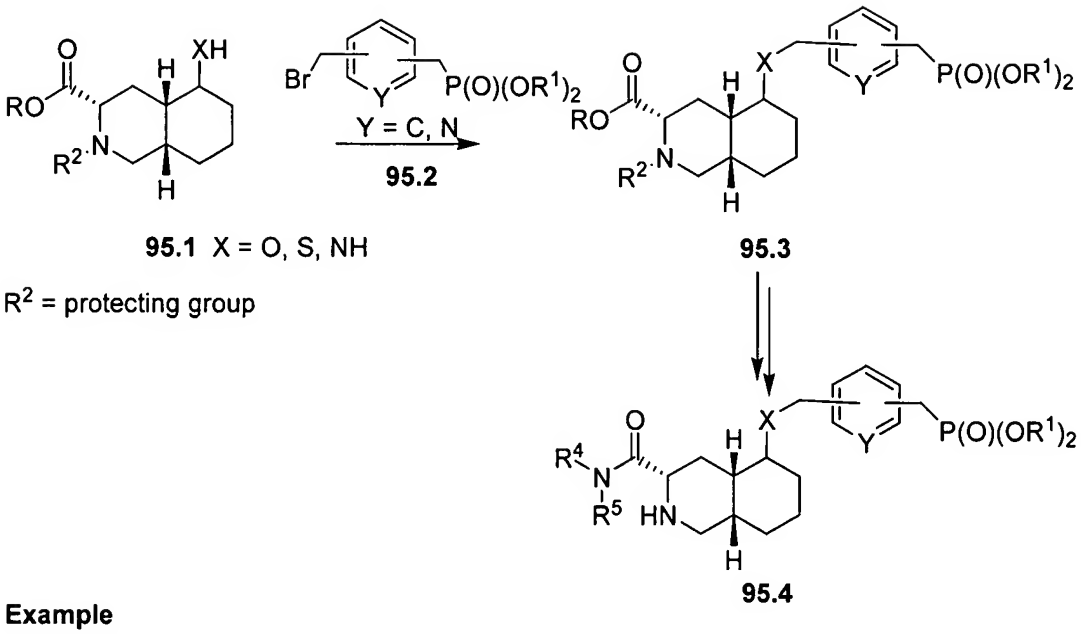
Using the above procedures, but employing, instead of the β-carbinol **95.5**, different carbinols, thiols or amines **95.1**, of either α- or β-orientation, and/or different phosphonates **95.2**, in place of the phosphonate **95.6**, there are obtained the corresponding products **95.4** in which the orientation of the side-chain is the same as that of the starting material **95.1**.

Schemes **92-95** illustrate the preparation of decahydroisoquinoline esters incorporating a phosphonate group linked to the decahydroisoquinoline nucleus.

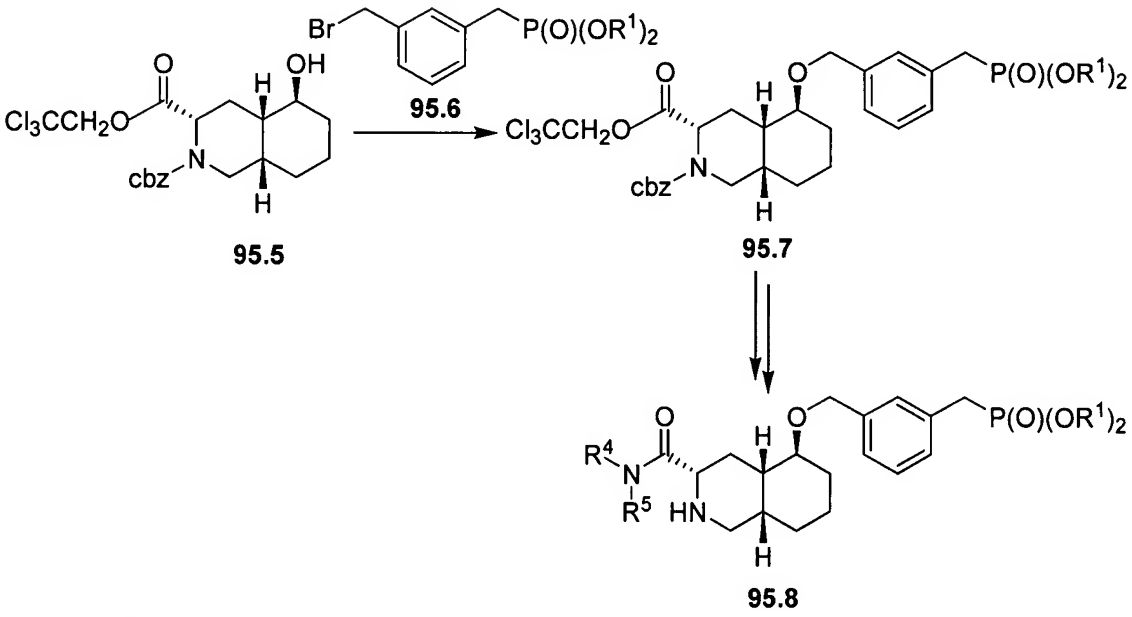
Scheme **96** illustrates the conversion of the latter group of compounds **96.1** (in which the group B is link-$P(O)(OR^1)_2$ or optionally protected precursor substituents thereto, such as, for example, OH, SH, NH_2) to the corresponding R^4R^5N amides **96.5**.

As shown in Scheme **96**, the ester compounds **96.1** are deprotected to form the corresponding carboxylic acids **96.2**. The methods employed for the deprotection are chosen based on the nature of the protecting group R, the nature of the N-protecting group R^2, and the nature of the substituent at the 6-position. For example, if R is trichloroethyl, the ester group is removed by treatment with zinc in acetic acid, as described in *J. Am. Chem. Soc.*, 88, 852, 1966. Conversion of the carboxylic acid **96.2** to the R^4R^5N amide **96.4** is then accomplished by reaction of the carboxylic acid, or an activated derivative thereof, with the amine R^4R^5NH **96.3** to afford the amide **96.4**, using the conditions described above for the preparation of the amide **1.6**. Deprotection of the NR^2 group, as described above, then affords the free amine **96.5**.

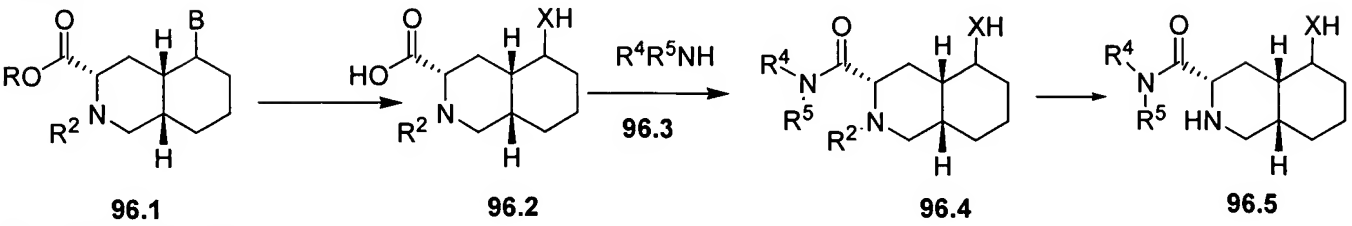
Scheme 95
Method



Example



Scheme 96
Method



Preparation of the phosphonate-containing tert. butylamides 37.1

Scheme **97** illustrates the preparation of the amides **37.1** in which the substituent A is either the group link $P(O)(OR^1)_2$ or a precursor thereto, such as [OH], [SH], Br etc, which are employed in the preparation of the intermediate phosphonate esters **10** (Schemes **37 – 40**). In this procedure, the BOC-protected decahydroisoquinoline carboxylic acid **97.1** is reacted with the tert. butylamine derivative **25.1**, in which the substituent A is the group link-$P(O)(OR^1)_2$, or a precursor group thereto, such as [OH], [SH], Br, etc, to afford the amide **97.2**. The reaction is conducted as described above for the preparation of the amides **1.3** and **1.6**. The BOC protecting group is then removed to yield the amine **37.1**.

Preparation of the phosphonate-containing thiazolidines 21.1

Schemes **98 - 101** illustrate the preparation of the thiazolidine derivatives **37.1**, in which the substituent A is either the group link $P(O)(OR^1)_2$ or a precursor thereto, such as [OH], [SH], Br etc, which are employed in the preparation of the intermediate phosphonate esters **6**. The preparation of the penicillamine analogs **98.5** in which R is alkyl is described in *J. Org. Chem.*, 1986, 51, 5153 and in *J. Labelled Comp. Radiochem.*, 1987, 24, 1265. The conversion of the penicillamine analogs **98.5** into the corresponding thiazolidines **98.7** is described in *J. Med. Chem.*, 1999, 42, 1789 and in *J. Med. Chem.*, 1989, 32, 466. The above-cited procedures, and their use to afford analogs of the thiazolidines **98.7** are shown in Scheme **98**.

In this procedure, a methyl ketone **98.2** is reacted with methyl isocyanoacetate **98.1** to afford the aminoacrylate product **98.3**. The condensation reaction is conducted in the presence of a base such as butyllithium or sodium hydride, in a solvent such as tetrahydrofuran at from -80° to 0°, to afford after treatment with aqueous ammonium chloride the N-formyl acrylate ester **98.3**. The latter compound is then reacted with phosphorus pentasulfide or Lawessons reagent and the like to yield the thiazoline derivative **98.4**. The reaction is performed in an aprotic solvent such as benzene, for example as described in *J. Org. Chem.*, 1986, 51, 5153. The thiazoline product **98.4** is then treated with dilute acid, for example dilute hydrochloric acid, to produce the aminothiol **98.5**. This compound is reacted with aqueous formaldehyde at pH 5, for example as described in *J. Med. Chem.*, 1999, 42, 1789, to prepare the thiazolidine **98.6**. The product is then converted, as described previously, into the BOC-protected analog **98.7**. Some examples of the use of the reactions of Scheme **98** for the preparation of functionally substituted thiazolidines **98.7** are shown below.

Scheme **98**, Example **1** illustrates the preparation of the BOC-protected hydroxymethyl thiazolidine **98.11**. In this procedure, methyl isocyanoacetate **98.1** is reacted with hydroxyacetone **98.8** in the presence of a base such as sodium hydride, to yield the aminoacrylate derivative **98.9**. The product is then reacted with phosphorus pentasulfide, as described above, to prepare the thiazoline **98.10**. The latter compound is then converted, as described above, into the thiazolidine derivative **98.11**.

Scheme **98**, Example **2**, depicts the preparation of bromophenyl-substituted thiazolidines **98.14**. In this reaction sequence, methyl isocyanoacetate **98.1** is condensed, as described above, with a bromoacetophenone **98.12** to give the aminocinnamate derivative **98.13**. The latter compound is then transformed, as described above, into the thiazolidine derivative **98.14**.

Scheme **98**, Example **3** depicts the preparation of the BOC-protected thiazolidine-5-carboxylic acid **98.18**. In this procedure, methyl isocyanoacetate **98.1** is reacted, as described above, with trichloroethyl pyruvate **98.15** to afford the aminoacrylate derivative **98.16**. This compound is then transformed, as described above, into the thiazolidine diester **98.17**. The trichloroethyl ester is then cleaved, for example by treatment with zinc in aqueous tetrahydrofuran at pH 4.2, as described in *J. Am. Chem. Soc.*, 88, 852, 1966, to afford the 5-carboxylic acid **98.18**.

Scheme **98**, Example **4**, depicts the preparation of the BOC-protected thiazolidine-4-carboxylic acid incorporating a phosphonate moiety. In this procedure, methyl isocyanoacetate **98.1** is condensed, as described above, with a dialkyl 2-oxopropyl phosphonate **98.19**, (Aldrich); the product **98.20** is then transformed, as described above, into the corresponding 4-carbomethoxythiazolidine. Hydrolysis of the methyl ester, for example by the use of one equivalent of lithium hydroxide in aqueous tetrahydrofuran, then yields the carboxylic acid **98.21**.

Scheme **99** illustrates the preparation of BOC-protected thiazolidine-4-carboxylic acids incorporating a phosphonate group attached by means of an oxygen atom and an alkylene chain. In this procedure, the hydroxymethyl thiazolidine **98.11** is reacted with a dialkyl bromoalkyl phosphonate **99.1** to afford the ether product **99.2**. The hydroxymethyl substrate **98.11** is treated in dimethylformamide solution with a strong base such as sodium hydride or lithium hexamethyldisilylazide, and an equimolar amount of the bromo compound **99.1** is added. The

product **99.2** is then treated with aqueous base, as described above, to effect hydrolysis of the methyl ester to yield the carboxylic acid **99.3**.

For example, the hydroxymethyl thiazolidine **98.11** is reacted with sodium hydride and a dialkyl bromoethyl phosphonate **99.4** (Aldrich) in dimethylformamide at 70°, to produce the phosphonate product **99.5**. Hydrolysis of the methyl ester then affords the carboxylic acid **99.6**.

Using the above procedures, but employing, in place of the dialkyl bromoethyl phosphonate **99.4**, different bromoalkyl phosphonates **99.1**, the corresponding products **99.3** are obtained.

Scheme **100** illustrates the preparation of BOC-protected thiazolidine-4-carboxylic acids incorporating a phosphonate group attached by means of a phenyl group. In this procedure, a bromophenyl-substituted thiazolidine **98.14** is coupled, as described above (Scheme **46**) in the presence of a palladium catalyst, with a dialkyl phosphite **100.1**, to produce the phenylphosphonate derivative **100.2**. The methyl ester is then hydrolyzed to afford the carboxylic acid **100.3**.

For example, the BOC-protected 5-(4-bromophenyl)thiazolidine **100.4** is coupled with a dialkyl phosphite **100.1** to yield the product **100.5**, which upon hydrolysis affords the carboxylic acid **100.6**.

Using the above procedures, but employing, in place of the 4-bromophenyl thiazolidine **100.4**, different bromophenyl thiazolidines **98.14**, the corresponding products **100.3** are obtained.

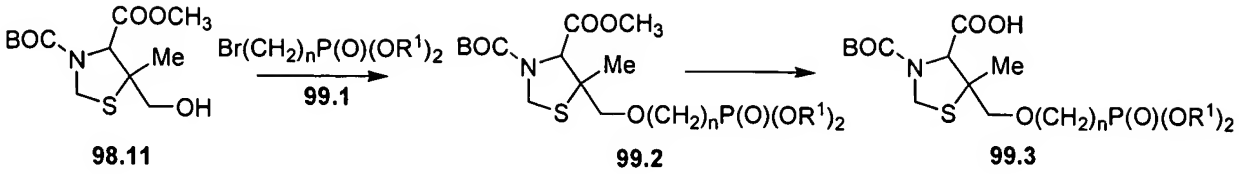
Scheme **101** illustrates the preparation of BOC-protected thiazolidine-4-carboxylic acids incorporating a phosphonate group attached by means of an amide linkage. In this procedure, a thiazolidine-5-carboxylic acid **98.18** is reacted with a dialkyl aminoalkyl phosphonate **101.1** to produce the amide **101.2**. The reaction is conducted as described above for the preparation of the amides **1.3** and **1.6**. The methyl ester is then hydrolyzed to afford the carboxylic acid **101.3**.

For example, the carboxylic acid **98.18** is reacted in tetrahydrofuran solution with an equimolar amount of a dialkyl aminopropyl phosphonate **101.4** (Acros) and dicyclohexylcarbodiimide, to afford the amide **101.5.** The methyl ester is then hydrolyzed to afford the carboxylic acid **101.6**.

Using the above procedures, but employing, in place of the dialkyl aminopropyl phosphonate **101.4**, different aminoalkyl phosphonates **101.1**, the corresponding products **101.3** are obtained.

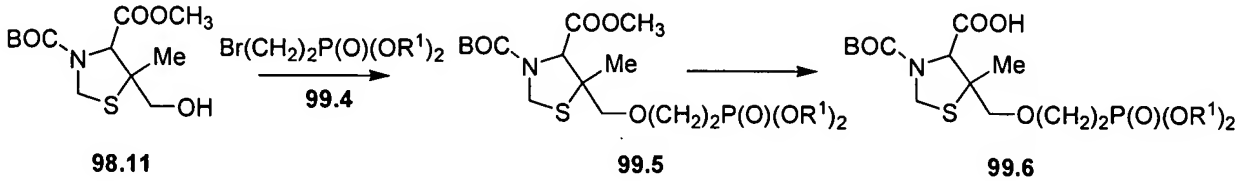
Scheme 99

Method



98.11 **99.2** **99.3**

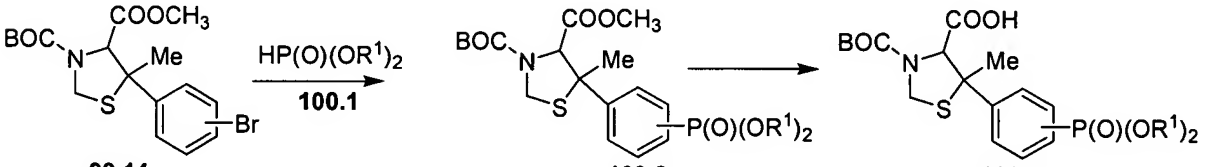
Example



98.11 **99.5** **99.6**

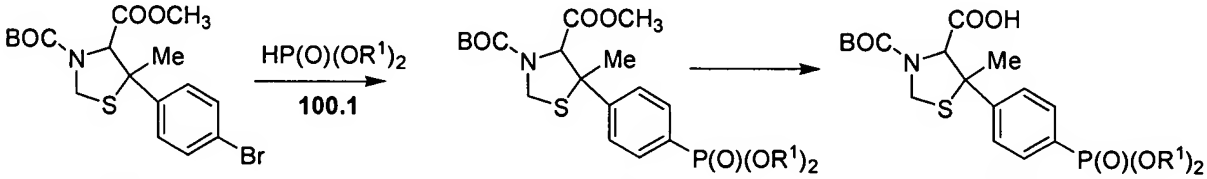
Scheme 100

Method



98.14 **100.2** **100.3**

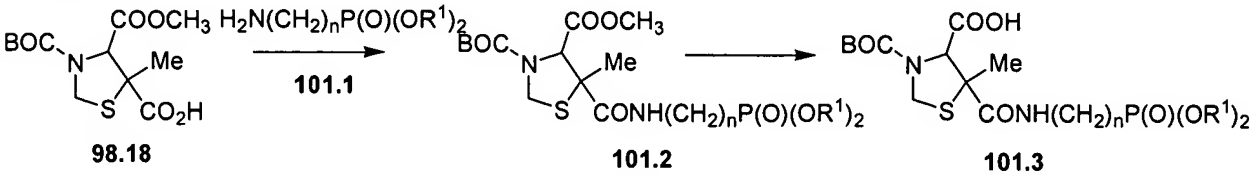
Example



100.4 **100.5** **100.6**

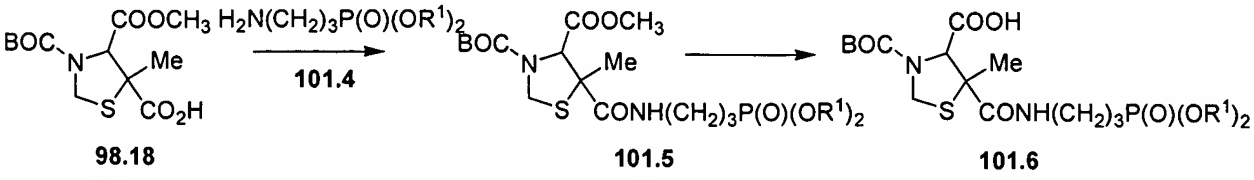
Scheme 101

Method



98.18 **101.2** **101.3**

Example



98.18 **101.5** **101.6**

Preparation of carbamates

The phosphonate esters **5 - 12** in which the R^8CO groups are formally derived from the carboxylic acids **C38 - C49** (Chart **2c**) contain a carbamate linkage. The preparation of carbamates is described in Comprehensive Organic Functional Group Transformations, A. R. Katritzky, ed., Pergamon, 1995, Vol. 6, p. 416ff, and in Organic Functional Group Preparations, by S. R. Sandler and W. Karo, Academic Press, 1986, p. 260ff.

Scheme **102** illustrates various methods by which the carbamate linkage can be synthesized. As shown in Scheme **102**, in the general reaction generating carbamates, a carbinol **102.1**, is converted into the activated derivative **102.2** in which Lv is a leaving group such as halo, imidazolyl, benztriazolyl and the like, as described herein. The activated derivative **102.2** is then reacted with an amine **102.3**, to afford the carbamate product **102.4**. Examples **1 – 7** in Scheme **102** depict methods by which the general reaction can be effected. Examples **8 - 10** illustrate alternative methods for the preparation of carbamates.

Scheme **102**, Example **1** illustrates the preparation of carbamates employing a chloroformyl derivative of the carbinol **102.5**. In this procedure, the carbinol **102.5** is reacted with phosgene, in an inert solvent such as toluene, at about 0°, as described in *Org. Syn. Coll.* Vol. 3, 167, 1965, or with an equivalent reagent such as trichloromethoxy chloroformate, as described in *Org. Syn. Coll.* Vol. 6, 715, 1988, to afford the chloroformate **102.6**. The latter compound is then reacted with the amine component **102.3**, in the presence of an organic or inorganic base, to afford the carbamate **102.7**. For example, the chloroformyl compound **102.6** is reacted with the amine **102.3** in a water-miscible solvent such as tetrahydrofuran, in the presence of aqueous sodium hydroxide, as described in *Org. Syn. Coll.* Vol. 3, 167, 1965, to yield the carbamate **102.7**. Alternatively, the reaction is performed in dichloromethane in the presence of an organic base such as diisopropylethylamine or dimethylaminopyridine.

Scheme **102**, Example **2** depicts the reaction of the chloroformate compound **102.6** with imidazole to produce the imidazolide **102.8**. The imidazolide product is then reacted with the amine **102.3** to yield the carbamate **102.7**. The preparation of the imidazolide is performed in an aprotic solvent such as dichloromethane at 0°, and the preparation of the carbamate is conducted in a similar solvent at ambient temperature, optionally in the presence of a base such as dimethylaminopyridine, as described in *J. Med. Chem.*, 1989, 32, 357.

Scheme **102** Example **3**, depicts the reaction of the chloroformate **102.6** with an activated hydroxyl compound R"OH, to yield the mixed carbonate ester **102.10**. The reaction is conducted in an inert organic solvent such as ether or dichloromethane, in the presence of a base such as dicyclohexylamine or triethylamine. The hydroxyl component R"OH is selected from the group of compounds **102.19 - 102.24** shown in Scheme **102**, and similar compounds. For example, if the component R"OH is hydroxybenztriazole **102.19**, N-hydroxysuccinimide **102.20**, or pentachlorophenol, **102.21**, the mixed carbonate **102.10** is obtained by the reaction of the chloroformate with the hydroxyl compound in an ethereal solvent in the presence of dicyclohexylamine, as described in *Can. J. Chem.*, 1982, 60, 976. A similar reaction in which the component R"OH is pentafluorophenol **102.22** or 2-hydroxypyridine **102.23** can be performed in an ethereal solvent in the presence of triethylamine, as described in *Synthesis*, 1986, 303, and *Chem. Ber.* 118, 468, 1985.

Scheme **102** Example **4** illustrates the preparation of carbamates in which an alkyloxycarbonylimidazole **102.8** is employed. In this procedure, a carbinol **102.5** is reacted with an equimolar amount of carbonyl diimidazole **102.11** to prepare the intermediate **102.8**. The reaction is conducted in an aprotic organic solvent such as dichloromethane or tetrahydrofuran. The acyloxyimidazole **102.8** is then reacted with an equimolar amount of the amine $R'NH_2$ to afford the carbamate **102.7**. The reaction is performed in an aprotic organic solvent such as dichloromethane, as described in *Tetrahedron Lett.*, 42, 2001, 5227, to afford the carbamate **102.7**.

Scheme **102**, Example **5** illustrates the preparation of carbamates by means of an intermediate alkoxycarbonylbenztriazole **102.13**. In this procedure, a carbinol ROH is reacted at ambient temperature with an equimolar amount of benztriazole carbonyl chloride **102.12**, to afford the alkoxycarbonyl product **102.13**. The reaction is performed in an organic solvent such as benzene or toluene, in the presence of a tertiary organic amine such as triethylamine, as described in *Synthesis*, 1977, 704. The product is then reacted with the amine $R'NH_2$ to afford the carbamate **102.7**. The reaction is conducted in toluene or ethanol, at from ambient temperature to about 80° as described in *Synthesis*, 1977, 704.

Scheme **102**, Example **6** illustrates the preparation of carbamates in which a carbonate $(R''O)_2CO$, **102.14**, is reacted with a carbinol **102.5** to afford the intermediate alkyloxycarbonyl intermediate **102.15**. The latter reagent is then reacted with the amine $R'NH_2$ to afford the

carbamate **102.7**. The procedure in which the reagent **102.15** is derived from hydroxybenztriazole **102.19** is described in *Synthesis*, 1993, 908; the procedure in which the reagent **102.15** is derived from N-hydroxysuccinimide **102.20** is described in *Tetrahedron Lett.*, 1992, 2781; the procedure in which the reagent **102.15** is derived from 2-hydroxypyridine **102.23** is described in *Tetrahedron Lett.*, 1991, 4251; the procedure in which the reagent **102.15** is derived from 4-nitrophenol **102.24** is described in *Synthesis* 1993, 103. The reaction between equimolar amounts of the carbinol ROH and the carbonate **102.14** is conducted in an inert organic solvent at ambient temperature.

Scheme **102**, Example **7** illustrates the preparation of carbamates from alkoxycarbonyl azides **102.16**. In this procedure, an alkyl chloroformate **102.6** is reacted with an azide, for example sodium azide, to afford the alkoxycarbonyl azide **102.16**. The latter compound is then reacted with an equimolar amount of the amine $R'NH_2$ to afford the carbamate **102.7**. The reaction is conducted at ambient temperature in a polar aprotic solvent such as dimethylsulfoxide, for example as described in *Synthesis*, 1982, 404.

Scheme **102**, Example **8** illustrates the preparation of carbamates by means of the reaction between a carbinol ROH and the chloroformyl derivative of an amine **102.17**. In this procedure, which is described in Synthetic Organic Chemistry, R. B. Wagner, H. D. Zook, Wiley, 1953, p. 647, the reactants are combined at ambient temperature in an aprotic solvent such as acetonitrile, in the presence of a base such as triethylamine, to afford the carbamate **102.7**.

Scheme **102**, Example **9** illustrates the preparation of carbamates by means of the reaction between a carbinol ROH and an isocyanate **102.18**. In this procedure, which is described in Synthetic Organic Chemistry, R. B. Wagner, H. D. Zook, Wiley, 1953, p. 645, the reactants are combined at ambient temperature in an aprotic solvent such as ether or dichloromethane and the like, to afford the carbamate **102.7**.

Scheme **102**, Example **10** illustrates the preparation of carbamates by means of the reaction between a carbinol ROH and an amine $R'NH_2$. In this procedure, which is described in *Chem. Lett.* 1972, 373, the reactants are combined at ambient temperature in an aprotic organic solvent such as tetrahydrofuran, in the presence of a tertiary base such as triethylamine, and selenium. Carbon monoxide is passed through the solution and the reaction proceeds to afford the carbamate **102.7**.

Interconversions of the phosphonates R-link-P(O)(OR^1)$_2$, R-link-P(O)(OR^1)(OH) and R-link-P(O)(OH)$_2$

Schemes **1** - **102** described the preparations of phosphonate esters of the general structure R-link-P(O)(OR^1)$_2$, in which the groups R^1, the structures of which are defined in Chart **1**, may be the same or different. The R^1 groups attached to a phosphonate esters **1** - **12**, or to precursors thereto, may be changed using established chemical transformations. The interconversions reactions of phosphonates are illustrated in Scheme **103**. The group R in Scheme **103** represents the substructure to which the substituent link-P(O)(OR^1)$_2$ is attached, either in the compounds **1** - **12** or in precursors thereto. The R^1 group may be changed, using the procedures described below, either in the precursor compounds, or in the esters **1** - **12**. The methods employed for a given phosphonate transformation depend on the nature of the substituent R^1. The preparation and hydrolysis of phosphonate esters is described in Organic Phosphorus Compounds, G. M. Kosolapoff, L. Maeir, eds, Wiley, 1976, p. 9ff.

The conversion of a phosphonate diester **103.1** into the corresponding phosphonate monoester **103.2** (Scheme **103**, Reaction **1**) can be accomplished by a number of methods. For example, the ester **103.1** in which R^1 is an aralkyl group such as benzyl, can be converted into the monoester compound **103.2** by reaction with a tertiary organic base such as diazabicyclooctane (DABCO) or quinuclidine, as described in *J. Org. Chem.*, 1995, 60, 2946. The reaction is performed in an inert hydrocarbon solvent such as toluene or xylene, at about 110°. The conversion of the diester **103.1** in which R^1 is an aryl group such as phenyl, or an alkenyl group such as allyl, into the monoester **103.2** can be effected by treatment of the ester **103.1** with a base such as aqueous sodium hydroxide in acetonitrile or lithium hydroxide in aqueous tetrahydrofuran. Phosphonate diesters **103.1** in which one of the groups R^1 is aralkyl, such as benzyl, and the other is alkyl, can be converted into the monoesters **103.2** in which R^1 is alkyl by hydrogenation, for example using a palladium on carbon catalyst. Phosphonate diesters in which both of the groups R^1 are alkenyl, such as allyl, can be converted into the monoester **103.2** in which R^1 is alkenyl, by treatment with chlorotris(triphenylphosphine)rhodium (Wilkinson's catalyst) in aqueous ethanol at reflux, optionally in the presence of diazabicyclooctane, for example by using the procedure described in *J. Org. Chem.*, 38, 3224, 1973 for the cleavage of allyl carboxylates.

The conversion of a phosphonate diester **103.1** or a phosphonate monoester **103.2** into the corresponding phosphonic acid **103.3** (Scheme **103**, Reactions **2** and **3**) can effected by reaction of the diester or the monoester with trimethylsilyl bromide, as described in *J. Chem. Soc.*, Chem. Comm., 739, 1979. The reaction is conducted in an inert solvent such as, for example, dichloromethane, optionally in the presence of a silylating agent such as bis(trimethylsilyl)trifluoroacetamide, at ambient temperature. A phosphonate monoester **103.2** in which R^1 is aralkyl such as benzyl, can be converted into the corresponding phosphonic acid **103.3** by hydrogenation over a palladium catalyst, or by treatment with hydrogen chloride in an ethereal solvent such as dioxan. A phosphonate monoester **103.2** in which R^1 is alkenyl such as, for example, allyl, can be converted into the phosphonic acid **103.3** by reaction with Wilkinson's catalyst in an aqueous organic solvent, for example in 15% aqueous acetonitrile, or in aqueous ethanol, for example using the procedure described in *Helv. Chim. Acta.*, 68, 618, 1985. Palladium catalyzed hydrogenolysis of phosphonate esters **103.1** in which R^1 is benzyl is described in *J. Org. Chem.*, 24, 434, 1959. Platinum-catalyzed hydrogenolysis of phosphonate esters **103.1** in which R^1 is phenyl is described in *J. Am. Chem. Soc.*, 78, 2336, 1956.

The conversion of a phosphonate monoester **103.2** into a phosphonate diester **103.1** (Scheme **103**, Reaction **4**) in which the newly introduced R^1 group is alkyl, aralkyl, haloalkyl such as chloroethyl, or aralkyl can be effected by a number of reactions in which the substrate **103.2** is reacted with a hydroxy compound R^1OH, in the presence of a coupling agent. Suitable coupling agents are those employed for the preparation of carboxylate esters, and include a carbodiimide such as dicyclohexylcarbodiimide, in which case the reaction is preferably conducted in a basic organic solvent such as pyridine, or (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PYBOP, Sigma), in which case the reaction is performed in a polar solvent such as dimethylformamide, in the presence of a tertiary organic base such as diisopropylethylamine, or Aldrithiol-2 (Aldrich) in which case the reaction is conducted in a basic solvent such as pyridine, in the presence of a triaryl phosphine such as triphenylphosphine. Alternatively, the conversion of the phosphonate monoester **103.2** to the diester **103.1** can be effected by the use of the Mitsonobu reaction, as described above (Scheme **47**). The substrate is reacted with the hydroxy compound R^1OH, in the presence of diethyl azodicarboxylate and a triarylphosphine such as triphenyl phosphine. Alternatively, the phosphonate monoester **103.2** can be transformed into the phosphonate diester **103.1**, in which

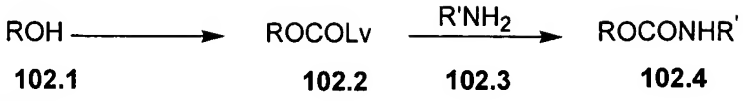
the introduced R^1 group is alkenyl or aralkyl, by reaction of the monoester with the halide R^1Br, in which R^1 is as alkenyl or aralkyl. The alkylation reaction is conducted in a polar organic solvent such as dimethylformamide or acetonitrile, in the presence of a base such as cesium carbonate. Alternatively, the phosphonate monoester can be transformed into the phosphonate diester in a two step procedure. In the first step, the phosphonate monoester **103.2** is transformed into the chloro analog $RP(O)(OR^1)Cl$ by reaction with thionyl chloride or oxalyl chloride and the like, as described in Organic Phosphorus Compounds, G. M. Kosolapoff, L. Maeir, eds, Wiley, 1976, p. 17, and the thus-obtained product $RP(O)(OR^1)Cl$ is then reacted with the hydroxy compound R^1OH, in the presence of a base such as triethylamine, to afford the phosphonate diester **103.1**.

A phosphonic acid R-link-$P(O)(OH)_2$ can be transformed into a phosphonate monoester $RP(O)(OR^1)(OH)$ (Scheme **103**, Reaction **5**) by means of the methods described above of for the preparation of the phosphonate diester R-link-$P(O)(OR^1)_2$ **103.1**, except that only one molar proportion of the component R^1OH or R^1Br is employed.

A phosphonic acid R-link-$P(O)(OH)_2$ **103.3** can be transformed into a phosphonate diester R-link-$P(O)(OR^1)_2$ **103.1** (Scheme **103**, Reaction **6**) by a coupling reaction with the hydroxy compound R^1OH, in the presence of a coupling agent such as Aldrithiol-2 (Aldrich) and triphenylphosphine. The reaction is conducted in a basic solvent such as pyridine. Alternatively, phosphonic acids **103.3** can be transformed into phosphonic esters **103.1** in which R^1 is aryl, by means of a coupling reaction employing, for example, dicyclohexylcarbodiimide in pyridine at ca 70°. Alternatively, phosphonic acids **103.3** can be transformed into phosphonic esters **103.1** in which R^1 is alkenyl, by means of an alkylation reaction. The phosphonic acid is reacted with the alkenyl bromide R^1Br in a polar organic solvent such as acetonitrile solution at reflux temperature, the presence of a base such as cesium carbonate, to afford the phosphonic ester **103.1**.

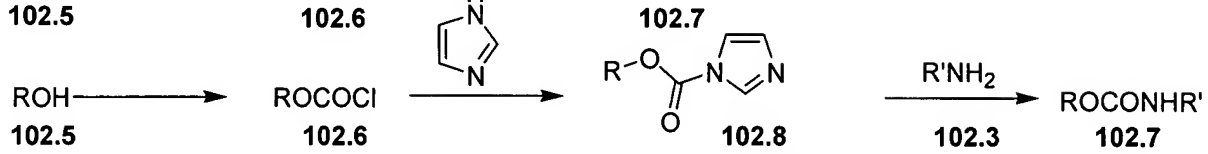
Scheme 102

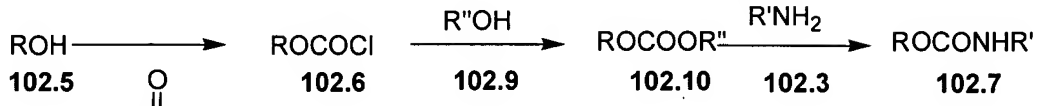
General reaction

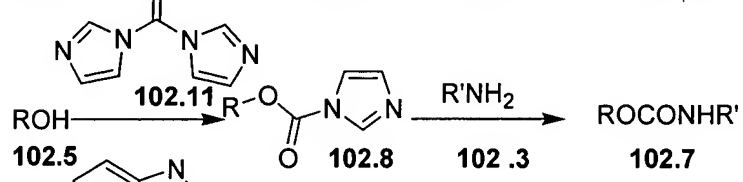


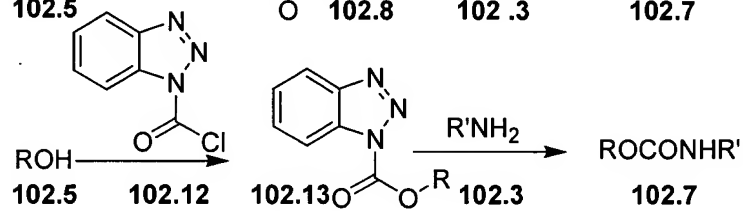
Examples

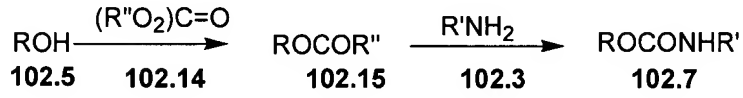
(1) 

(2) 

(3) 

(4) 

(5) 

(6) 

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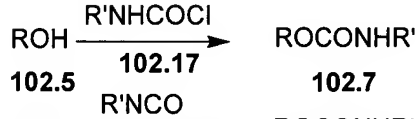
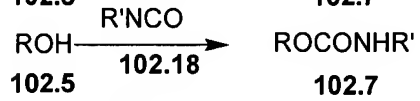
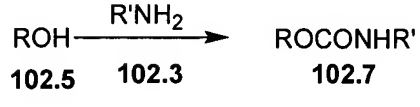
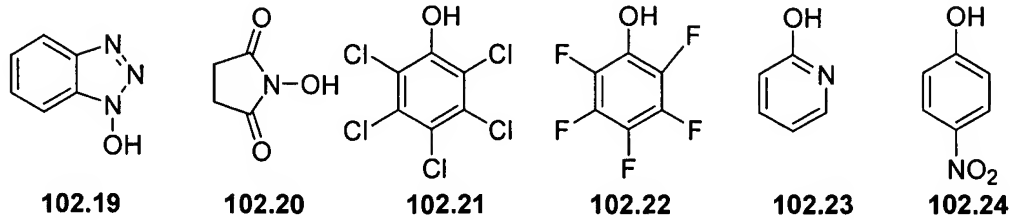
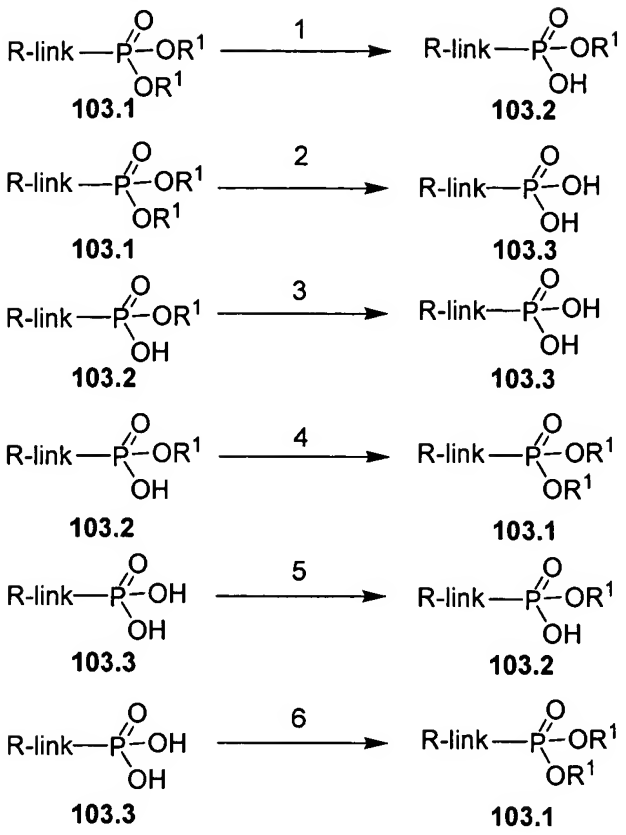
(8) 

(9) 

(10) 

R"OH = 

Scheme 103



General applicability of methods for introduction of phosphonate substituents

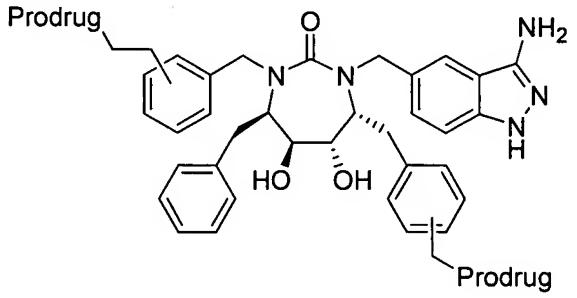
The procedures described herein for the introduction of phosphonate moieties (Schemes **45 - 101**) are, with appropriate modifications known to one skilled in the art, transferable to different chemical substrates. Thus, the methods described above for the introduction of phosphonate groups into hydroxymethyl benzoic acids (Schemes **45 - 52**) are applicable to the introduction of phosphonate moieties into the dimethoxyphenol, quinoline, phenylalanine, thiophenol, tert. butylamine, benzylamine, decahydroisoquinoline or thiazolidine substrates, and the methods described herein for the introduction of phosphonate moieties into the dimethoxyphenol, quinoline, phenylalanine, thiophenol, tert. butylamine, benzylamine, decahydroisoquinoline or thiazolidine substrates, (Schemes **53 - 101**) are applicable to the introduction of phosphonate moieties into carbinol substrates.

Preparation of phosphonate intermediates 11 and 12 with phosphonate moieties incorporated into the groups R^8CO and $R^{10}R^{11}N$

The chemical transformations described in Schemes **1 - 103** illustrate the preparation of compounds **1 -10** in which the phosphonate ester moiety is attached to the benzoic acid moiety, (Schemes **46 - 52**), the dimethylphenol moiety (Schemes **53 - 56**), the quinoline carboxamide moiety (Schemes **57 - 61**), the 5-hydroxyisoquinoline moiety (Schemes **62 - 66**), the phenylalanine moiety (Schemes **67 - 71**), the thiophenol moiety, (Schemes **72 - 83**), the tert. butylamine moiety, (Schemes **84 - 87**), the benzylamine moiety, (Schemes **88 - 90**), the decahydroisoquinoline moiety, (Schemes **91 - 97**) or the thiazolidine moiety, (Schemes **98 - 101**). The various chemical methods employed for the preparation of phosphonate groups can, with appropriate modifications known to those skilled in the art, be applied to the introduction of a phosphonate ester group into the compounds R^8COOH and $R^{10}R^{11}NH$, as defined in Charts **3a**, **3b**, **3c** and **4**. The resultant phosphonate-containing analogs, designated as $R^{8a}COOH$ and $R^{10a}R^{11a}NH$ can then, using the procedures described above, be employed in the preparation of the compounds **11** and **12**. The procedures required for the utilization of the phosphonate-containing analogs $R^{8a}COOH$ and $R^{10a}R^{11a}NH$ are the same as those described above for the utilization of the R^8COOH and $R^{10}R^{11}NH$ reactants.

<u>Examples For The Preparation Of Cyclic Carbonyl-Like Phosphonate Protease Inhibitors (CCPPI)</u>

Phosphonamidate Prodrugs



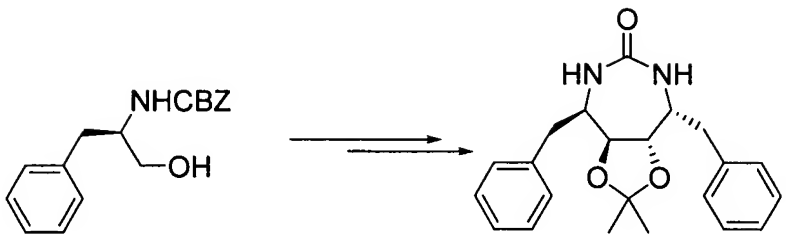
Scheme 1-2	Scaffold Synthesis
Scheme 3-10	P2'-Benzyl ether phosphonates
Scheme 11-13	P2'-Alkyl ether phosphonates

Scheme 14-17 P2'-Benzyl Amide phosphonates

Scheme 18-25 P1-Phosphonates

Scheme 50 Reagents

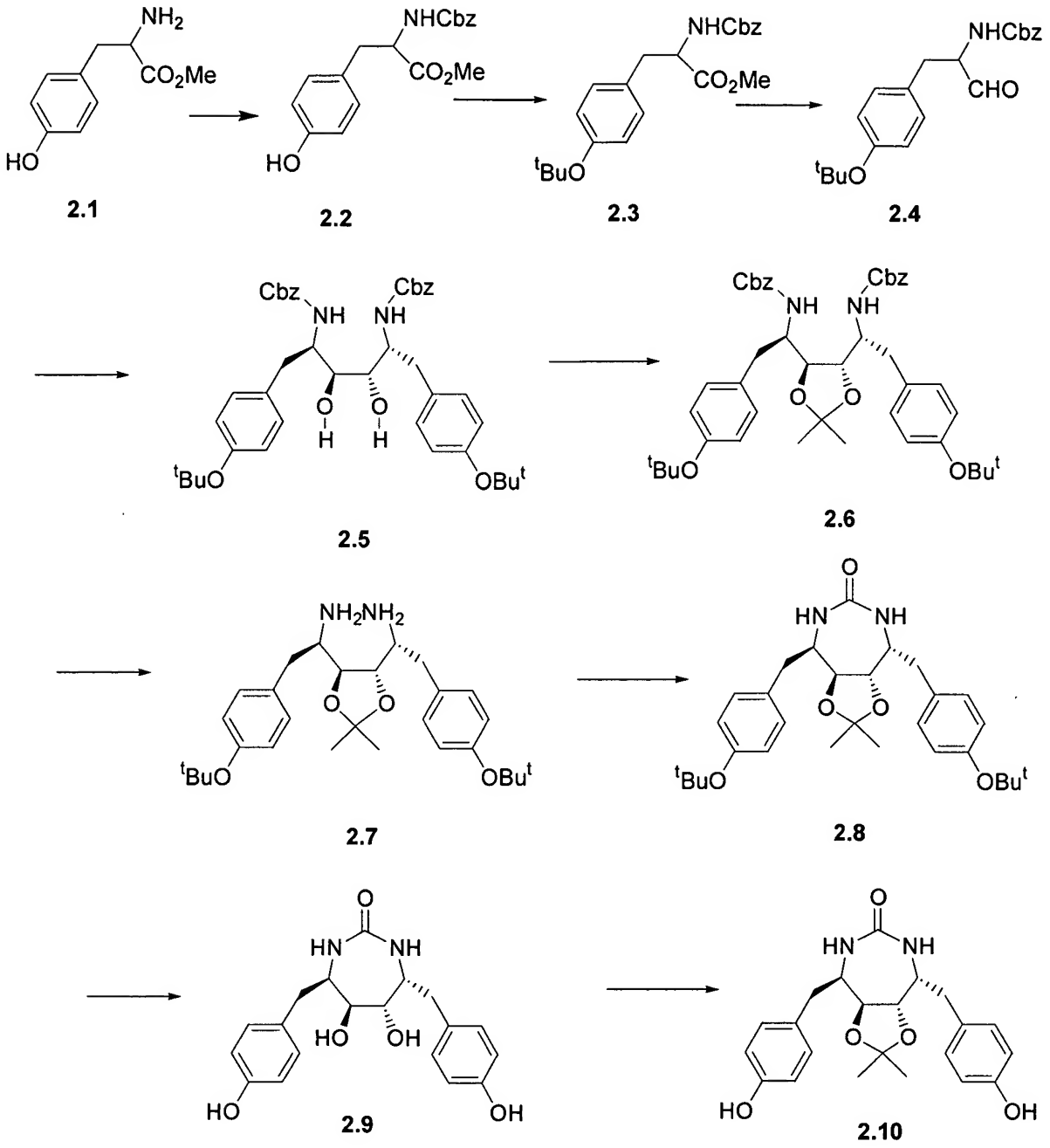
Scheme 1



1 **1.1**

The conversion of **1** to **1.1** is described in *J. Org Chem* 1996, 61, p444-450

Scheme 2



2-Benzyloxycarbonylamino-3-(4-tert-butoxy-phenyl)-propionic acid methyl ester (2.3)

H-D-Tyr-O-me hydrochloride **2.1** (25 g, 107.7 mmol) is dissolved in methylene chloride (150 mL) and aqueous sodium bicarbonate (22 g in 150 mL water), and then cooled to 0°C. To this resulting solution benzyl chloroformate (20 g, 118 mmol) is slowly added. After complete addition, the resulting solution is warmed to room temperature ,and is then stirred for 2 h. The

organic phase is separated, dried over Na_2SO_4, and concentrated under reduced pressure, to give the crude carbamate **2.2** (35g). The crude CBZ-Tyr-OMe product is dissolved in methylene chloride (300 mL) containing concentrated H_2SO_4. Isobutene is bubbled though the solution for 6 h. The reaction is then cooled to 0°C, and neutralized with saturated $NaHCO_3$ aqueous solution. The organic phase is separated, dried, concentrated under reduced pressure, and purified by silica gel column chromatography to afford the tert-butyl ether **2.3** (25.7 g, 62 %).

[2-(4-tert-Butoxy-phenyl)-1-formyl-ethyl]-carbamic acid benzyl ester (2.4)
(Reference J. O. C. 1997, 62, 3884)

To a stirred –78°C methylene chloride solution (60 mL) of **2.3,** DIBAL (82 mL of 1.5 M in toluene, 123 mmol) was added over 15 min. The resultant solution was stirred at –78°C for 30 min. Subsequently, a solution of EtOH/36 % HCl (9/1; 15 mL) is added slowly. The solution is added to a vigorously stirred aqueous HCl solution (600 mL, 1N) at 0°C. The layers are then separated, and the aqueous phase is extracted with cold methylene chloride. The combined organic phases are washed with cold 1N HCl aqueous solution, water, dried over Na_2SO_4, and then concentrated under reduced pressure to give the crude aldehyde **2.4** (20 g, 91 %).

[4-Benzyloxycarbonylamino-1-(4-tert-butoxy-benzyl)-5-(4-tert-butoxy-phenyl)-2,3-dihydroxy-pentyl]-carbamic acid benzyl ester (2.5)

To a slurry of $VCl_3(THF)_3$ in methylene chloride (150 mL) at room temperature is added Zinc powder (2.9 g, 44 mmol), and the resulting solution is then stirred at room temperature for 1 hour. A solution of aldehyde **2.4** (20 g, 56 mmol) in methylene chloride (100 mL) is then added over 10 min. The resulting solution is then stirred at room temperature overnight, poured into an ice-cold H_2SO_4 aqueous solution (8 mL in 200 mL), and stirred at 0°C for 30 min. The methylene chloride solution is separated, washed with 1N HCl until the washing solution is light blue. The organic solution is then concentrated under reduced pressure (solids are formed during concentration), and diluted with hexane. The precipitate is collected and washed thoroughly with a hexane/methylene chloride mixture to give the diol product **2.5**. The filtrate is concentrated under reduced pressure and subjected to silica gel chomatography to afford a further 1.5 g of **2.5**. (Total = 13 g, 65 %).

[1-{5-[1-Benzyloxycarbonylamino-2-(4-tert-butoxy-phenyl)-ethyl]-2,2-dimethyl-[1,3]dioxolan-4-yl}-2-(4-tert-butoxy-phenyl)-ethyl]-carbamic acid benzyl ester (2.6)

Diol **2.5** (5 g, 7 mmol) is dissolved in acetone (120 mL), 2,2-dimethoxypropane (20 mL), and pyridinium p-toluenesulfonate (120 mg, 0.5 mmol). The resulting solution is refluxed for 30 min., and then concentrated under reduced pressure to almost dryness. The resulting mixture is partitioned between methylene chloride and saturated $NaHCO_3$ aqueous solution, dried, concentrated under reduced pressure, and purified by silica gel column chomatography to afford isopropylidene protected diol **2.6** (4.8 g, 92 %).

4,8-Bis-(4-tert-butoxy-benzyl)-2,2-dimethyl-hexahydro-1,3-dioxa-5,7-diaza-azulen-6-one (2.8)

The diol **2.6** is dissolved in EtOAc/EtOH (10 mL/2 mL) in the presence of 10 % Pd/C and hydrogenated at atmospheric pressure to afford the diamino compound **2.7**. To a solution of crude **2.7** in 1,1,2,2-tetrachloroethane is added 1,1-carboxydiimidazole (1.05 g, 6.5 mmol) at room temperature. The mixture is stirred for 10 min, and the resulting solution is then added dropwise to a refluxing 1,1',2,2'-tetrachloroethane solution (150 mL). After 30 min., the reaction mixture is cooled to room temperature, and washed with 5 % citric acid aqueous solution, dried over Na_2SO4, concentrated under reduced pressure, and purified by silica gel column chomatography to afford the cyclourea derivative **2.8** (1.92 g, 60 % over 2 steps).

5,6-Dihydroxy-4,7-bis-(4-hydroxy-benzyl)-[1,3]diazepan-2-one (2.9)

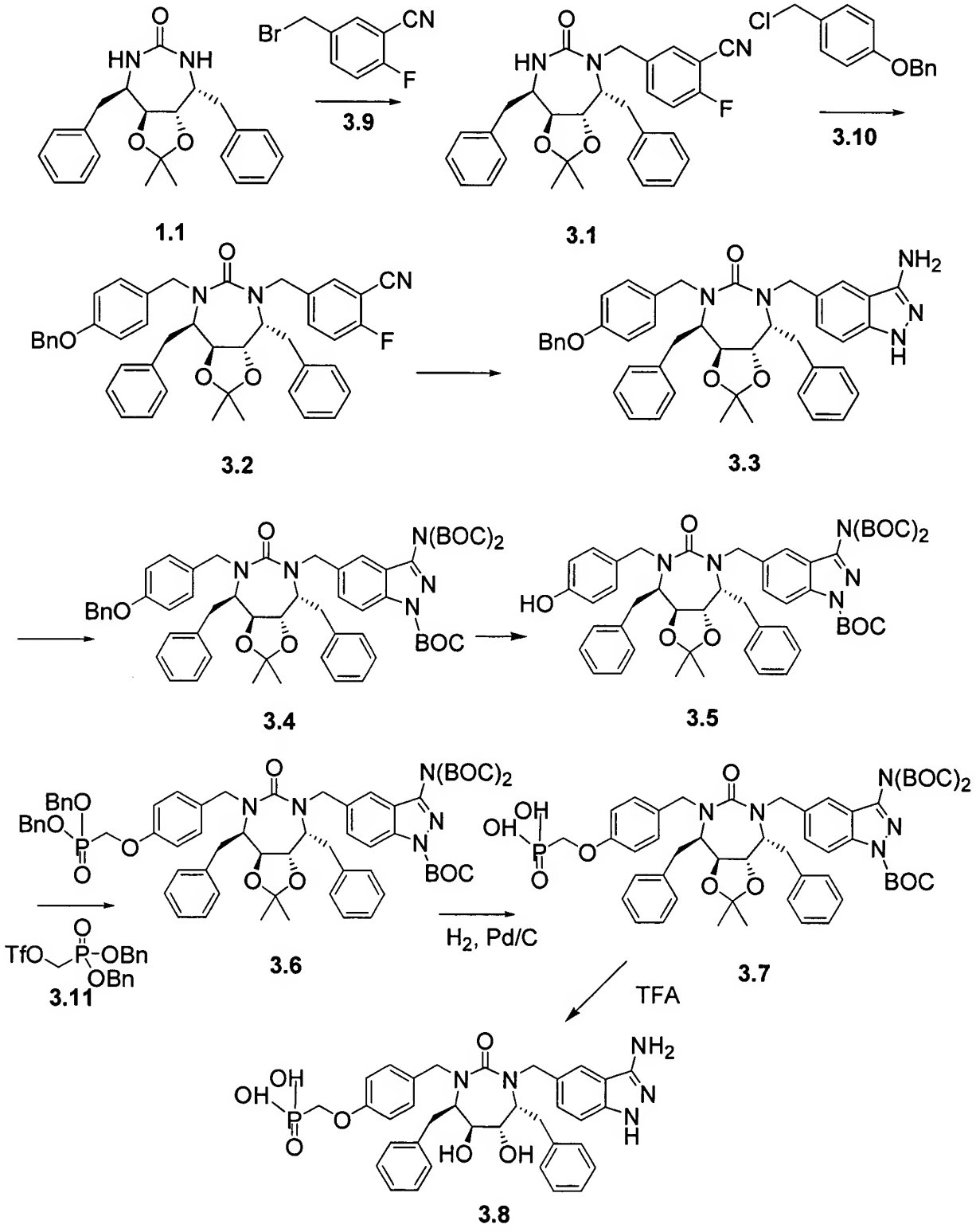
Cyclic Urea **2.8** (0.4 g, 0.78 mmol) was dissolved in dichloromethane (3 mL) and treated with TFA (1 mL). The mixture was stirred at room temperature for 2 h upon which time a white solid precipitated. 2 drops of water and methanol (2 mL) were added and the homogeneous solution was stirred for 1 h and concentrated under reduced pressure. The crude solid, **2.9**, was dried overnight and then used without further purification.

4,8-Bis-(4-hydroxy-benzyl)-2,2-dimethyl-hexahydro-1,3-dioxa-5,7-diaza-azulen-6-one (2.10)

Diol **2.9** (1.8 g, 5.03 mmol) was dissolved in DMF (6 mL) and 2,2-dimethoxypropane (12 mL). P-TsOH (95 mg) was added and the mixture stirred at 65°C for 3 h. A vacuum was applied to remove water and then the mixture was stirred at 65°C for a further 1 h. The excess

dimethoxypropane was then distilled and the remaining DMF solution was then allowed to cool. The solution of acetonide **2.10** can then used without further purification in future reactions.

Scheme 3



3-Cyano-4-fluorobenzyl urea 3.1: A solution of urea **1.1** (1.6 g, 4.3 mmol) in THF was treated with sodium hydride (0.5 g of 60 % oil dispersion, 13 mmol). The mixture was stirred at room temperature for 30 min and then treated with 3-cyano-4-fluorobenzyl bromide **3.9** (1.0 g, 4.8 mmol). The resultant solution was stirred at room temperature for 3 h, concentrated under reduced pressure, and then partitioned between CH_2Cl_2 and saturated brine solution containing 1 % citric acid. The organic phase was separated , dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 15-25% ethyl acetate in hexanes to yield urea **3.1** (1.5 g, 69 %) as a white form.

Benzyl ether 3.2: A solution of **3.1** (0.56 g, 1.1 mmol) in DMF (5 mL) was treated with sodium hydride (90 mg of 60 % oil dispersion, 2.2 mmol) and the resultant mixture stirred at room temperature for 30 min. 4-Benzyloxy benzyl chloride **3.10** (0.31 g, 1.3 mmol) was added and the resultant solution stirred at room temperature for 3 h. The mixture was concentrated under reduced pressure and then partitioned between CH_2Cl_2 and saturated brine solution. The organic phase was separated, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by silica gel eluting with 1-10% ethyl acetate in hexanes to yield compound **3.2** (0.52 g, 67 %) as white form.

Indazole 3.3: Benzyl ether **3.2** (0.51 g, 0.73 mmol) was dissolved in n-butanol (10 mL) and treated with hydrazine hydrate (1 g, 20 mmol). The mixture was refluxed for 4 h and then allowed to cool to room temperature. The mixture was concentrated under reduced pressure and the residue was then partitioned between CH_2Cl_2 and 10 % citric acid solution. The organic phase was separated, concentrated under reduced pressure, and then purified by silica gel column eluting with 5% methanol in CH_2Cl_2 to afford indazole **3.3** (0.42 g, 82 %) as white solid.

Boc-indazole 3.4: A solution of indazole **3.3** (0.4 g, 0.59 mmol) in CH_2Cl_2 (10 mL) was treated with diisopropylethylamine (0.19 g, 1.5 mmol), DMAP (0.18 g, 1.4 mmol), and di-tert-butyl dicarbonate (0.4 g, 2 mmol). The mixture was stirred at room temperature for 3 h and then partitioned between CH_2Cl_2 and 5 % citric acid solution. The organic phase was separated, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 2% methanol in CH_2Cl_2 to afford **3.4** (0.42 g, 71 %).

Phenol 3.5: A solution of **3.4** (300 mg, 0.3 mmol) in ethyl acetate (10 mL) and methanol (10 mL) was treated with 10 % Pd/C (40 mg) and stirred under a hydrogen atmosphere (balloon)

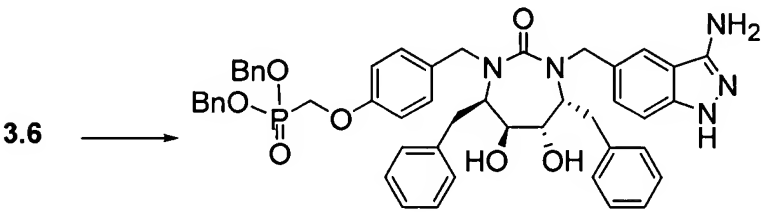
for 16 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to yield **3.5** as a white powder. This was used without further purification.

Dibenzyl ester 3.6: A solution of **3.5** (0.1 mmol) in THF (5 mL) was treated with dibenzyl triflate **3.11** (90 mg, 0.2 mmol), and cesium carbonate (0.19 g, 0.3 mmol). The mixture was stirred at room temperature for 4 h and then concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and saturated brine. The organic phase was separated, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 20-40% ethyl acetate in hexanes to afford **3.6** (70 mg, 59 %). ^{1}H NMR ($CDCl_3$): δ 8,07 (d, 1H), 7.20-7.43 (m, 16H), 7.02-7.15 (m, 8 H), 6.80 (d, 2H), 5.07-5.18 (m, 4H), 5.03 (d, 1H), 4.90 (d, 1H), 4.20 (d, 2H), 3.74-3.78 (m, 4H), 3.20 (d, 1H), 3.05 (d, 1H) 2.80-2.97 (m, 4H), 1.79 (s, 9H), 1.40 (s, 18H), 1.26 (s, 6H); ^{31}P NMR ($CDCl_3$): 20.5 ppm.

Phosphonic acid 3.7: A solution of dibenzylphosphonate **3.6** (30 mg) in EtOAc (10 mL) was treated with 10% Pd/C (10 mg) and the mixture was stirred under a hydrogen atmosphere (balloon) for 3 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to afford phosphonic acid **3.7**. This was used without further purification.

Phosphonic acid 3.8: The crude phosphonic acid **3.7** was dissolved in CH_2Cl_2 (2 mL) and treated with trifluoroacetic acid (0.4 mL). The resultant mixture was stirred at room temperature for 4 h. The mixture was concentrated under reduced pressure and then purified by preparative HPLC (35 % CH_3CN/65 % H_2O) to afford the phosphonic acid **3.8** (9.4 mg, 55 %). ^{1}H NMR (CD_3OD): δ 7.71 (s, 1H), 7.60 (d, 1H), 6.95-7.40 (m, 15H), 4.65 (d, 2H), 4.17 (d, 2H), 3.50-3.70 (m, 3H), 3.42 (d, 1H), 2.03-3.14 (m, 6H); ^{31}P NMR ($CDCl_3$): 17.30.

Scheme 4

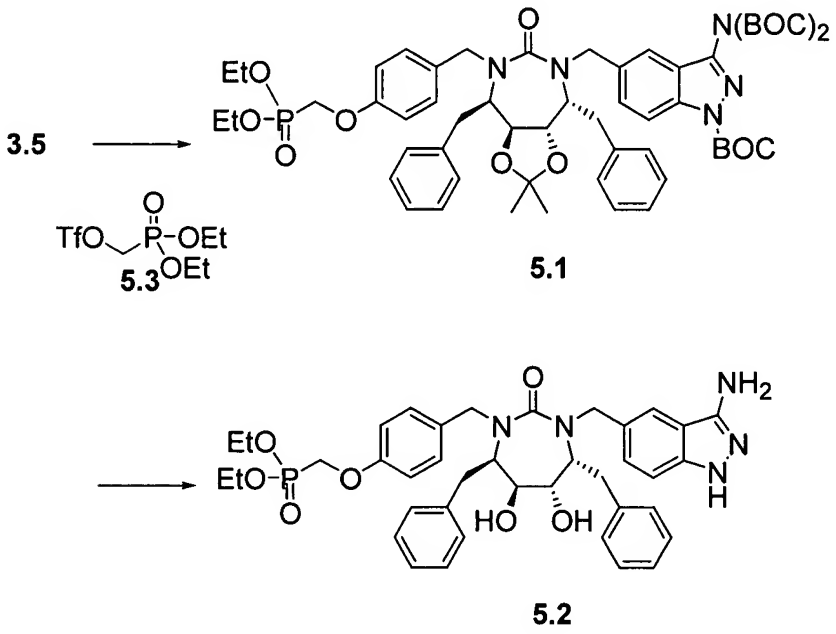


4.1

Dibenzylphosphonate 4.1: A solution of **3.6** (30 mg, 25 μmol) in CH_2Cl_2 (2 mL) was treated with TFA (0.4 mL) and the resultant mixture was stirred at room temperature for 4 h. The mixture was concentrated under reduced pressure and the residue was purified by silica gel

eluting with 50% ethyl acetate in hexanes to afford **4.1** (5 mg, 24%). ^{1}H NMR ($CDCl_3$): δ 6.96-7.32 (m, 25H), 6.95 (d, 2H), 5.07-5.18 (m, 4H), 4.86 (d, 1H), 4.7 5 (d, 1H), 4.18 (d, 2H), 3.40-3.62 (m, 4H), 3.25 (d, 1H), 2.80-3.15 (m, 6H); ^{31}P NMR ($CDCl_3$) 20.5 ppm; MS : 852 (M + H), 874 (M + Na).

Scheme 5

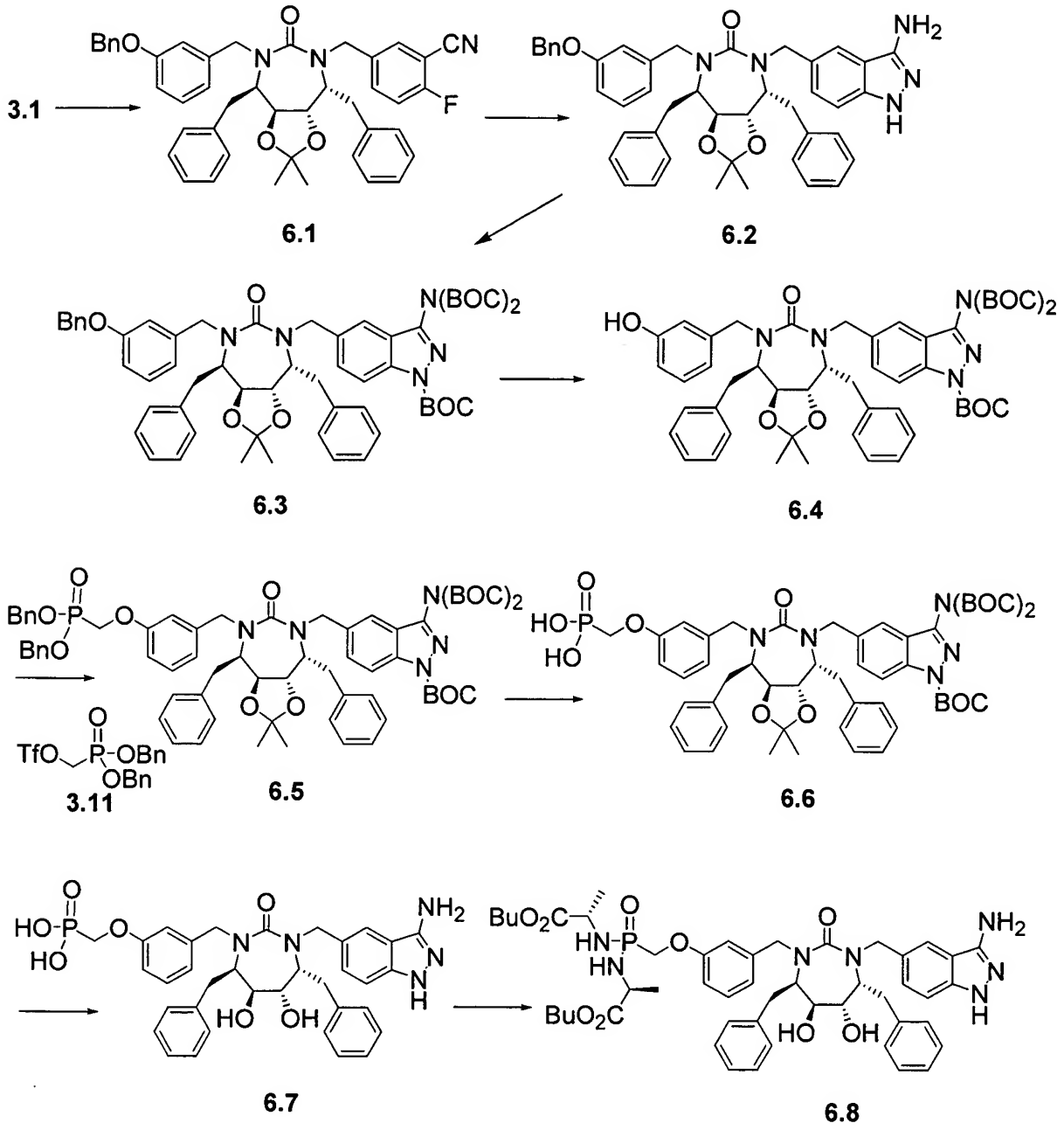


Diethylphosphonate 5.1: A solution of phenol **3.5** (48 mg, 52 μmol) in THF (5 mL) was treated with triflate **5.3** (50 mg, 165 μmol), and cesium carbonate (22 mg, 0.2 mmol). The resultant mixture was stirred at room temperature for 5 h and then concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and saturated brine. The organic phase was separated, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 7% methanol in CH_2Cl_2 to afford **5.1** (28 mg, 50 %). ^{1}H NMR ($CDCl_3$): δ 8,06 (d, 1H), 7.30-7.43 (m, 7H), 7.02-7.30 (m, 7 H), 6.88 (d, 2H), 5.03 (d, 1H), 4.90 (d, 1H), 4.10-4.25 (m, 6H), 3.64-3.80 (m, 4H), 3.20 (d, 1H), 3.05 (d, 1H) 2.80-2.97 (m, 4H), 1.79 (s, 9H), 1.20-1.50 (m, 30H); ^{31}P NMR ($CDCl_3$): 18.5 ppm; MS :1068 (M + H), 1090 (M + Na).

Diethylphosphonate 5.2: A solution of **5.1** (28 mg, 26 μmol) in CH_2Cl_2 (2 mL) was treated with TFA (0.4 mL) and the resultant mixture was stirred at room temperature for 4 hrs. The mixture was concentrated under reduced pressure and the residue was purified by silica gel

to afford **5.2** (11 mg, 55 %). ^{1}H NMR ($CDCl_3$ + 10 % CD_3OD): δ 6.96-7.35 (m, 15H), 6.82 (d, 2H), 4.86(d, 1H), 4.75 (d, 1H), 4.10-4.23 (M, 6H), 3.40-3.62 (m, 4H), 2.80-3.20 (m), 1.31 (t, 6 H); ^{31}P NMR ($CDCl_3$ + 10 % CD_3OD): 19.80 ppm; MS : 728 (M + H).

Scheme 6



3-Benzyloxybenzyl urea 6.1: The urea **3.1** (0.87 g, 1.7 mmol) was dissolved in DMF and treated with sodium hydride (60% dispersion, 239 mg, 6.0 mmol) followed by m-

benzyloxybenzylbromide **6.9** (0.60 g, 2.15 mmol). The mixture was stirred for 5 h and then diluted with ethyl acetate. The solution was washed with water, brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 25% ethyl acetate in hexanes to afford urea **6.1** (0.9 g, 75%).

Indazole 6.2: The urea **6.1** (41 mg, 59 μmol) was dissolved in n-butanol (1.5 mL) and treated with hydrazine hydrate (100 μL, 100 mmol). The mixture was refluxed for 2 h and then allowed to cool. The mixture was diluted with ethyl acetate, washed with 10% citric acid solution, brine, saturated $NaHCO_3$, and finally brine again. The organic phase was dried over sodium sulfate, filtered and concentrated under reduced pressure to give the crude product **6.2** (35 mg, 83%). (*Chem. Biol.* 1998, 5, 597-608).

Boc-indazole 6.3: The indazole **6.2** (1.04 g, 1.47 mmol) was dissolved in CH_2Cl_2 (20 mL) and treated with di-t-butyl dicarbonate (1.28 g, 5.9 mmol), DMAP (0.18 g, 1.9 mmol) and DIPEA (1.02 ml, 9.9 mmol). The mixture was stirred for 3 h and then diluted with ethyl acetate. The solution was washed with 5% citric acid solution, $NaHCO_3$, brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 50% ethyl acetate in hexanes to give **6.3** (0.71 g, 49%).

Phenol 6.4 : Compound **6.3** (20 mg, 0.021 mmol) was dissolved in MeOH (1 mL) and EtOAc (1 mL) and treated with 10% Pd/ C catalyst (5 mg). The mixture was stirred under a hydrogen atmosphere (balloon) until completion. The catalyst was removed by filtration and the filtrate concentrated under reduced pressure to afford compound **6.4** (19 mg, 100%).

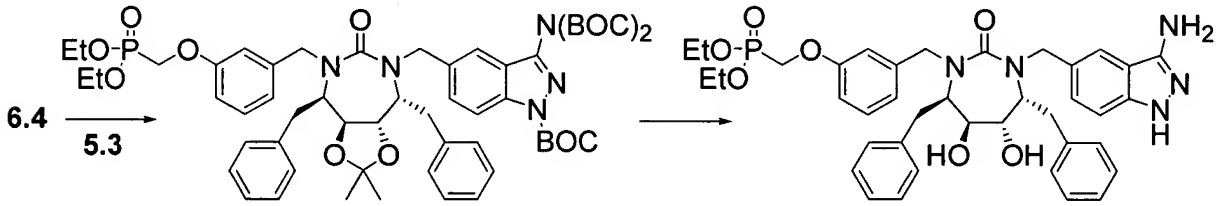
Dibenzyl phosphonate 6.5: A solution of compound **6.4** (0.34 g, 0.37 mmol) in acetonitrile (5 mL) was treated with Cs_2CO_3 (0.36 g, 1.1 mmol) and triflate **3.11** (0.18 mL, 0.52 mmol). The reaction mixture was stirred for 1 h. The reaction mixture was filtered and the filtrate was then concentrated under reduced pressure. The residue was re-dissolved in EtOAc, washed with water, saturated $NaHCO_3$, and finally brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with hexane: EtOAc (1:1) to afford compound **6.5** (0.32 g, 73%).

Phosphonic acid 6.6: Compound **6.5** (208 mg, 0.174 mmol) was treated in the same manner as benzyl phosphonate **3.6** in the preparation of phosphonate diacid **3.7**, except MeOH was used as the solvent, to afford compound **6.6** (166 mg, 94%).

Phosphonic acid 6.7: Compound **6.6** (89 mg, 0.088 mmol) was treated according to the conditions described in Scheme 3 for the conversion of **3.7** into **3.8**. The residue was purified by preparative HPLC eluting with a gradient of 90% methanol in 100 mM TEA bicarbonate buffer and 100% TEA bicarbonate buffer to afford phosphonic acid **6.7** (16 mg, 27%).

Bisamidate 6.8: Triphenylphosphine (112 mg, 0.43 mmol) and aldrithiol-2 (95 mg, 0.43 mmol) were mixed in dry pyridine (0.5 mL). In an adjacent flask the diacid **6.7** (48 mg, 0.71 mmol) was suspended in dry pyridine (0.5 mL) and treated with DIPEA (0.075 mL 0.43 mmol) and L-AlaButyl ester hydrochloride (78 mg, 0.43 mmol) and finally the triphenylphosphine, aldrithiol-2 mixture. The reaction mixture was stirred under nitrogen for 24 h then concentrated under reduced pressure. The residue was purified by preparative HPLC eluting with a gradient of 5% to 95% acetonitrile in water. The product obtained was then further purified by silica gel eluting with CH_2Cl_2 : MeOH (9:1) to give compound **6.8** (9 mg, 14%).

Scheme 7

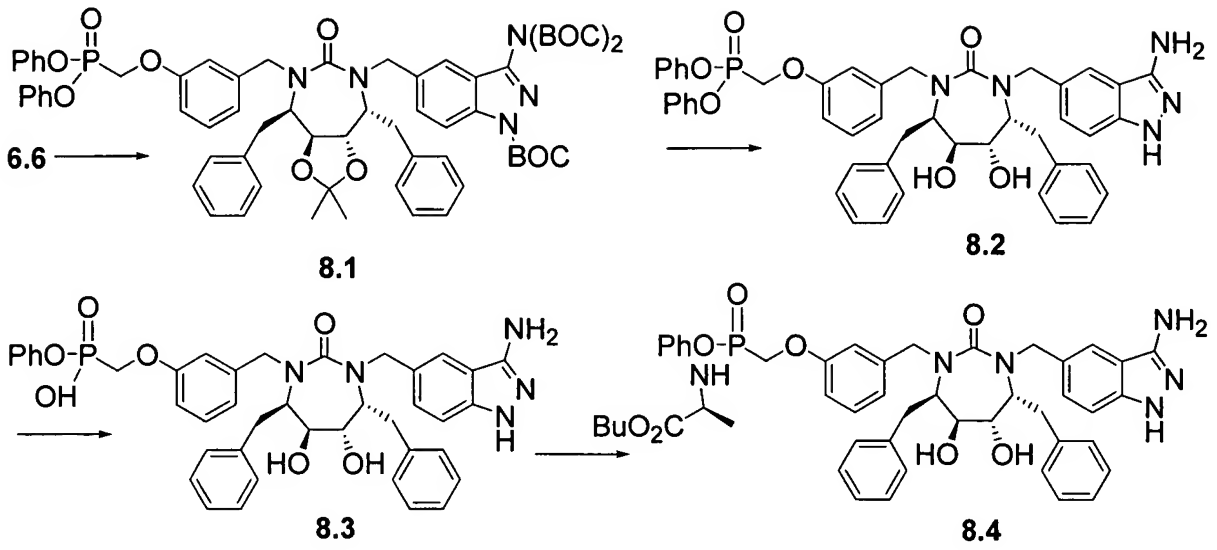


7.1 **7.2**

Diethyl phosphonate 7.1: Compound **6.4** (164 mg, 0.179 mmol) was treated according to the procedure used to generate compound **6.5** except triflate **5.3** was used in place of triflate **3.11** to afford compound **7.1** (142 mg, 74%).

Diethylphosphonate 7.2: Compound **7.1** (57 mg, 0.053 mmol) was treated according to the conditions used to form **6.7** from **6.6**. The residue formed was purified by silica gel eluting with CH_2Cl_2 : MeOH (9:1) to afford compound **7.2** (13 mg, 33%).

Scheme 8



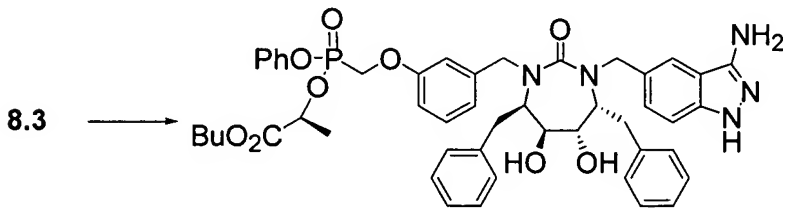
Diphenylphosphonate 8.1: A solution of **6.6** (0.67g, 0.66 mmol) in pyridine (10 mL) was treated with phenol (0.62 g, 6.6 mmol) and DCC (0.82 mg, 3.9 mmol). The resultant mixture was stirred at room temperature for 5 min and then the solution was heated at 70°C for 3 h. The mixture was allowed to cool to room temperature and then diluted with EtOAc and water (2 mL). The resultant mixture was stirred at room temperature for 30 min and then concentrated under reduced pressure. The residue was triturated with CH_2Cl_2, and the white solid that formed was removed by filtration. The filtrate was concentrated under reduced pressure and the resultant residue was purified by silica gel eluting with 30% ethyl acetate in hexanes to yield **8.1** (0.5 g, 65 %). ^{1}H NMR ($CDCl_3$): δ 8,08 (d, 1H), 7.41 (d, 1H), 7.05-7.35 (m, 22H), 6.85 (d, 2H), 6.70 (s, 1H). 5.19 (d, 1H), 5.10 (d, 1H), 4.70 (d, 2H), 3.70-3.90 (m, 4H), 3.20 (d, 1H), 3.11 (d, 1H), 2.80-2.97 (m, 4H), 1.79 (s, 9H), 1.40 (s, 18H), 1.30 (s, 6H); ^{31}P NMR ($CDCl_3$): 12.43 ppm.

Diphenylphosphonate 8.2: A solution of **8.1** (0.5 g, 0.42 mmol) in CH_2Cl_2 (4 mL) was treated with TFA (1 mL) and the resultant mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and azeotroped twice with CH_3CN. The residue was purified by silica gel eluting with 5% methanol in CH_2Cl_2 to afford diphenylphosphonate **8.2** (0.25 g, 71 %). ^{1}H NMR ($CDCl_3$): δ 7.03-7.40 (m, 21H), 6.81-6.90 (m, 3H), 4.96 (d, 1H), 4.90 (d, 1H) 4.60-4.70 (m, 2H), 3.43-3.57 (m, 4H), 3.20 (d, 1H), 2.80-2.97 (m, 5H); ^{31}P NMR ($CDCl_3$): 12.13 ppm; MS : 824 (M + H).

Monophenol 8.3: The monophenol **8.3** (124 mg, 68 %) was prepared from the diphenol **8.2** by treating with 1N NaOH in acetonitrile at 0°C.

Monoamidate 8.4: To a pyridine solution (0.5 mL) of **8.3** (40 mg, 53 μmol), n-butyl amidate HCl salt (116 mg, 640 μmol), and DIPEA (83 mg, 640 μmol) was added a pyridine solution (0.5 mL) of triphenyl phosphine (140 mg, 640 μmol), and aldrithiol-2 (120 mg, 640 μmol). The resulting solution was stirred at 65°C overnight, worked up, and purified by preparative TLC twice to give **8.4** (1.8 mg). δ 4.96 (d, 1H), 4.90 (d, 1H) 4.30-4.6 (m, 2H), 3.9-4.2 (m, 2H), 3.6-3.70 (m, 4H), 3.2-3.3 (d, 1H), 2.80-3.1 (m, 4H); MS: 875 (M + H) & 897 (M + Na).

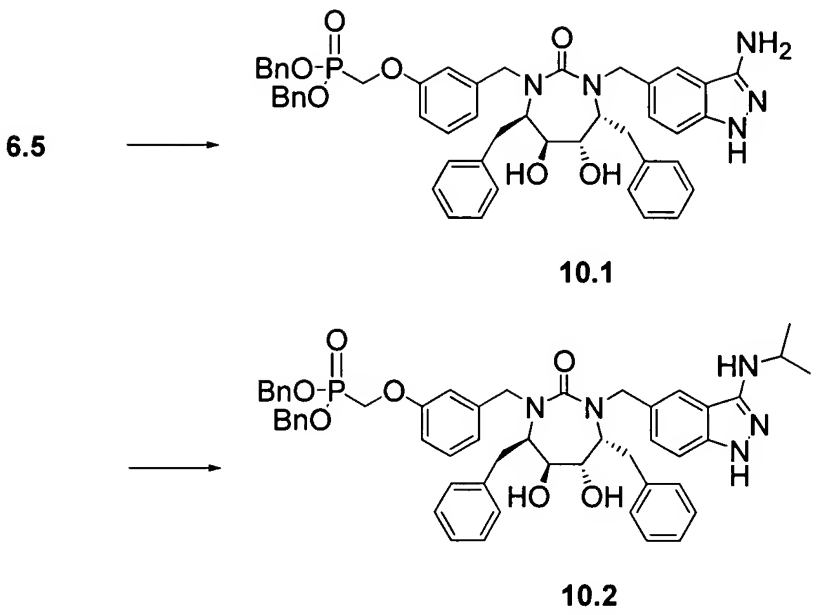
Scheme 9



9.1

Monolactate 9.1: The monolactate **9.1** is prepared from **8.3** using the conditions described above for the preparation of the monoamidate **8.4** except n-butyl lactate was used in place of n-butyl amidate HCl salt.

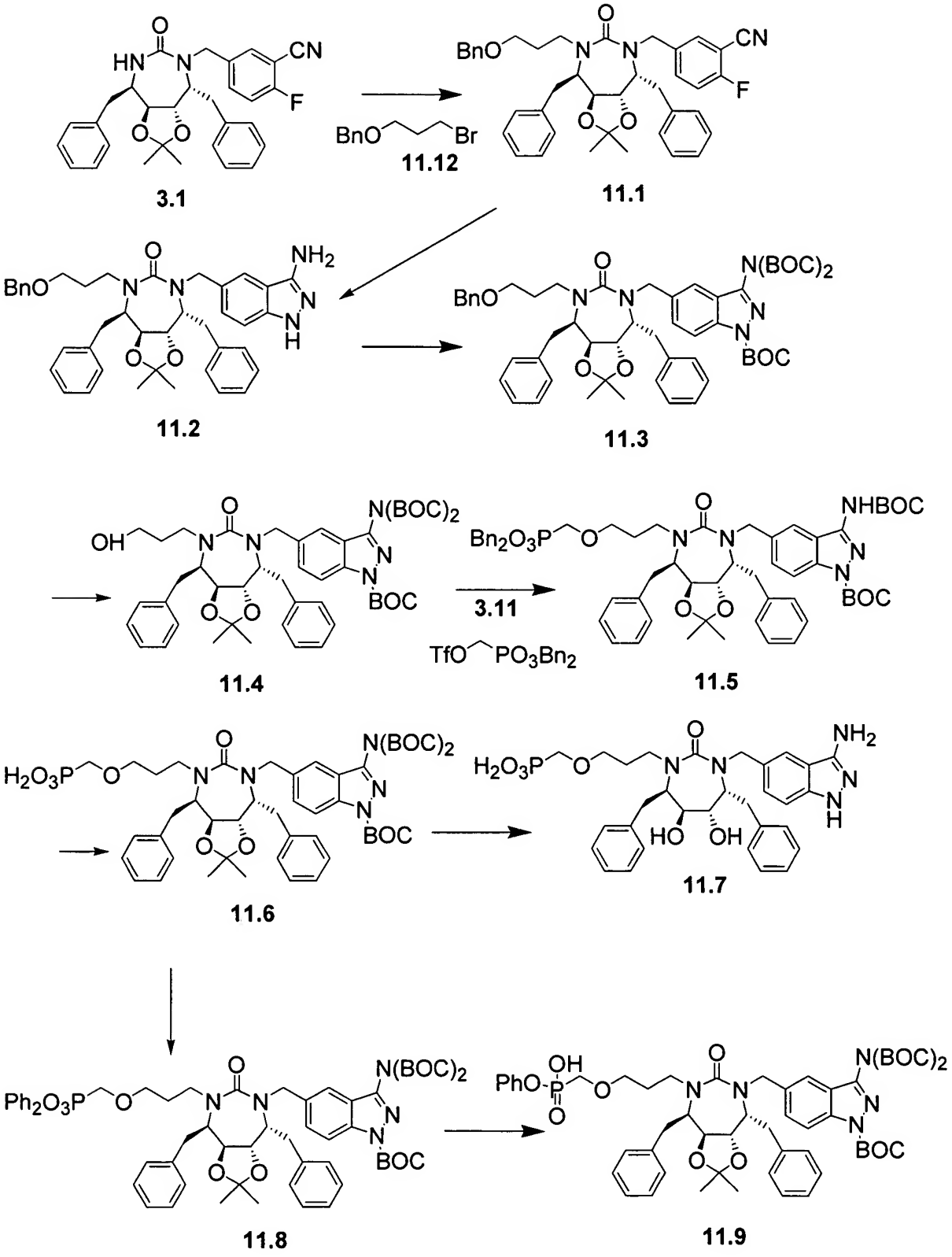
Scheme 10

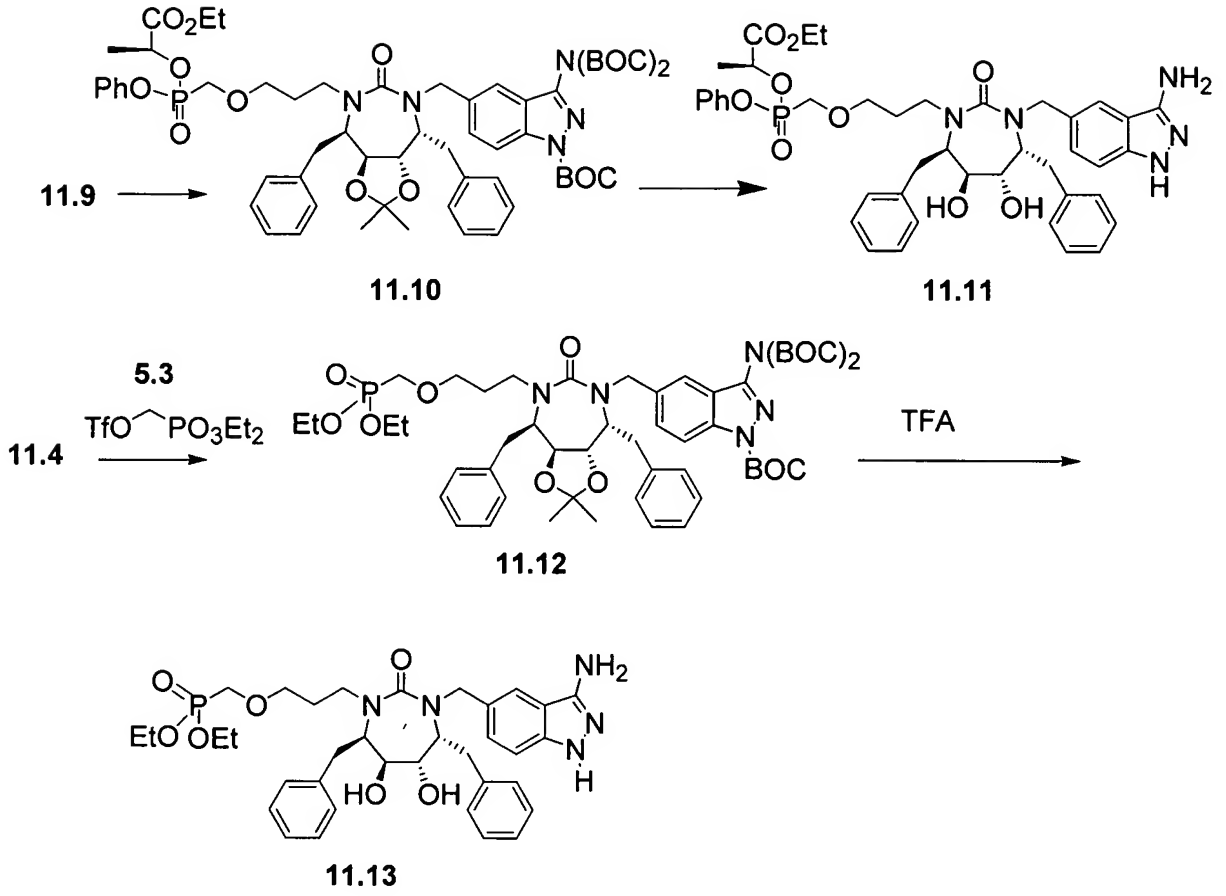


Dibenzylphosphonate 10.1: Compound **6.5** (16 mg, 0.014 mmol) was dissolved in CH_2Cl_2 (2 mL) and cooled to 0°C. TFA (1 mL) was added and the reaction mixture was stirred for 0.5 h. The mixture was then allowed to warm to room temperature for 2 h. The reaction mixture was concentrated under reduced pressure and azeotroped with toluene. The residue was purified by silica gel eluting with CH_2Cl_2 : MeOH (9:1) to afford compound **10.1** (4 mg, 32%).

Isopropylamino indazole 10.2 : Compound **10.1** (30 mg, 0.35 mmol) was treated with acetone according to the method of Henke *et al.* (*J. Med. Chem.* 40 17 (1997) 2706-2725) to yield **10.2** as a crude residue. The residue was purified by silica gel eluting with CH_2Cl_2 : MeOH (93:7) to afford compound **10.2** (3.4 mg, 10%).

Scheme 11





Benzyl ether 11.1: A DMF solution (5 mL) of **3.1** (0.98 g, 1.96 mmol) was treated with NaH (0.24 g of 60 % oil dispersion, 6 mmol) for 30 min, followed by the addition of sodium iodide (0.3 g, 2 mmol), and benzoxypropyl bromide (0.55 g, 2.4 mmol). After the reaction for 3 h at room temperature, the reaction mixture was partitioned between methylene chloride and saturated NaCl, dried, and purified to give **11.1** (0.62 g, 49 %).

Aminoindazole 11.2: A n-butanol solution (10 mL) of **11.1** (0.6 g, 0.92 mmol) and hydrazine hydrate (0.93 g, 15.5 mmol) was heated at reflux for 4 h. The reaction mixture was concentrated under reduced pressure to give crude **11.2** (~0.6 g).

Tri-BOC-Aminoindazole 11.3: A methylene chloride solution (10 mL) of crude **11.2**, DIPEA (0.36 g, 2.8 mmol), $(BOC)_2O$ (0.73 g, 3.3 mmol), and DMAP (0.34 g, 2.8 mmol) was stirred for 5 h at room temperature, partitioned between methylene chloride and 5 % citric acid solution, dried, purified by silica gel column chomatography to give **11.3** (0.51 g, 58 %, 2 steps).

3-Hydroxypropyl cyclic urea 11.4: An ethyl acetate/ethanol solution (30 mL/5 mL) of **11.3** (0.5 g, 0.52 mmol) was hydrogenated at 1 atm in the presence of 10 % Pd/C (0.2 g) for 4 h.

The catalyst was removed by filtration. The filtrate was then concentrated under reduced pressure to afford crude **11.4** (0.44 g, 98 %).

Dibenzyl phosphonate 11.5: A THF solution (3 mL) of **11.4** (0.5 g, 0.57 mmol) and triflate dibenzyl phosphonate **3.11** (0.37 g, 0.86 mmol) was cooled to –3°C, followed by addition of n-BuLi (0.7 mL of 2.5 M hexane solution, 1.7 mmol). After 2 h reaction, the reaction mixture was partitioned between methylene chloride and saturated NaCl solution, concentrated under reduced pressure. The residue was redissolved in methylene chloride (10 mL), and reacted with $(BOC)_2O$ (0.15 g, 0.7 mmol) in the presence of DMAP (0.18 g, 0.57 mmol), DIPEA (0.18 g, 1.38 mmol) for 2 h at room temperature. The reaction mixture was worked up, and purified by silica gel chromatography to give **11.5** (0.25 g, 43 %).

Phosphonic diacid 11.7: An ethyl acetate solution (2 mL) of **11.5A** (11 mg, 10.5 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (10 mg) for 6 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give crude **11.6**. The crude **11.6** was redissolved in methylene chloride (1 mL) and treated with TFA (0.2 mL) for 4 h at room temperature. The reaction mixture was concentrated under reduced pressure and purified by HPLC to give **11.7** (2 mg, 30%).

NMR (CD_3OD): δ 7.1-7.3 (m, 11H), 7.0-7.1 (d, 2H), 4.95 (d, 1H), 3.95-4.1 (d, 1H), 2.9 - 3.3 (m, 4H), 2.3-2.45 (m, 1H), 1.6-1.8 (m, 2H). P NMR (CD_3OD):15.5 ppm. MS: 624 (M + 1).

Diphenyl phosphonate 11.8: A pyridine solution (1 mL) of **11.6** (0.23 g, 0.23 mmol), phenol (0.27 g, 2.8 mmol), and DCC (0.3 g, 1.4 mmol) was stirred for 5 min. at room temperature, then reacted at 70°C for 3 h. The reaction mixture was cooled to room temperature, concentrated under reduced pressure, and purified by silica gel column chromatograph to afford **11.8** (0.11g, 41 %).

Monophenyl phosphonate 11.9: An acetonitrile solution (2 mL) of **11.8** (0.12 g, 0.107 mmol) at 0°C was treated with 1N sodium hydroxide aqueous solution (0.2 mL) for 1.5 h., then acidified with Dowex (50wx8-200, 120 mg). The Dowex was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was triturated with 10 % EtOAc/90 % hexane twice to afford **11.9** (90 mg, 76 %) as a white solid.

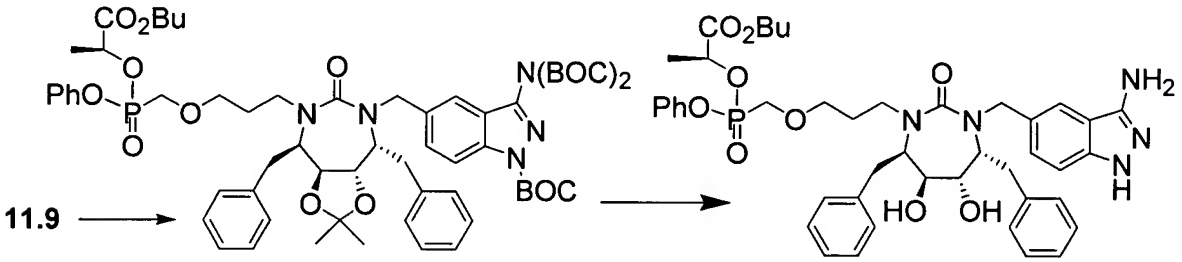
Mono-ethyl lactate phosphonate 11.10: A pyridine solution (0.3 mL) of **11.9** (33 mg, 30 μmol), ethyl lactate (41 mg, 340 μmol), and DCC (31 mg, 146 μmol) was stirred at room temperature for 5 min, then reacted at 70°C for 1.5 h. The reaction mixture was concentrated

under reduced pressure, partitioned between methylene chloride and saturated NaCl solution, and purified by silica gel chromatography to give **11.10** (18 mg, 50 %).

Ethyl lactate phosphonate 11.11: A methylene chloride solution (0.8 mL) of **11.10** (18 mg, 15.8 μmol) was treated with TFA (0.2 mL) for 4 h, and then concentrated under reduced pressure. The residue was purified by preparative TLC to give **11.11** (6 mg, 50 %). NMR ($CDCl_3$ + ~10 %CD_3OD): δ 7.0-7.3 (m, 16 H), 6.8-7.0 (m, 2H), 4.9-5.0 (m, 1H), 4.75 (d, 1H), 4.1-4.2 (m, 2H). 3.5-4.0 (m, 10H), 2.18-2.3. (m, 1H), 1.6-1.7 (m, 1), 1.47 & 1.41 (2d, 3H), 1.22 (t, 3H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 19.72 & 17.86 ppm.

Diethyl phosphonate 11.13: Compound **11.13** (6 mg) was prepared as described above in Scheme 5 from **11.4** (30 mg, 34 μmol) and triflate phosphonate **5.3** (52 mg, 172 μmol), followed by TFA treatment. NMR ($CDCl_3$ + ~10 %CD_3OD): δ 7.1-7.32 (m, 11 H), 6.9-7.0 (d, 2H), 4.75 (d, 1H), 4.1-4.2 (2q, 4H), 3.84-3.9 (m, 1H), 3.4-3.8 (m, 8H), 2.7-3.1 (m, 4H), 2.1-2.5 (m, 1H), 1.5-1.7 (m, 2H), 1.25-1.35 (2t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 21.63 ppm. MS: 680 (M + 1).

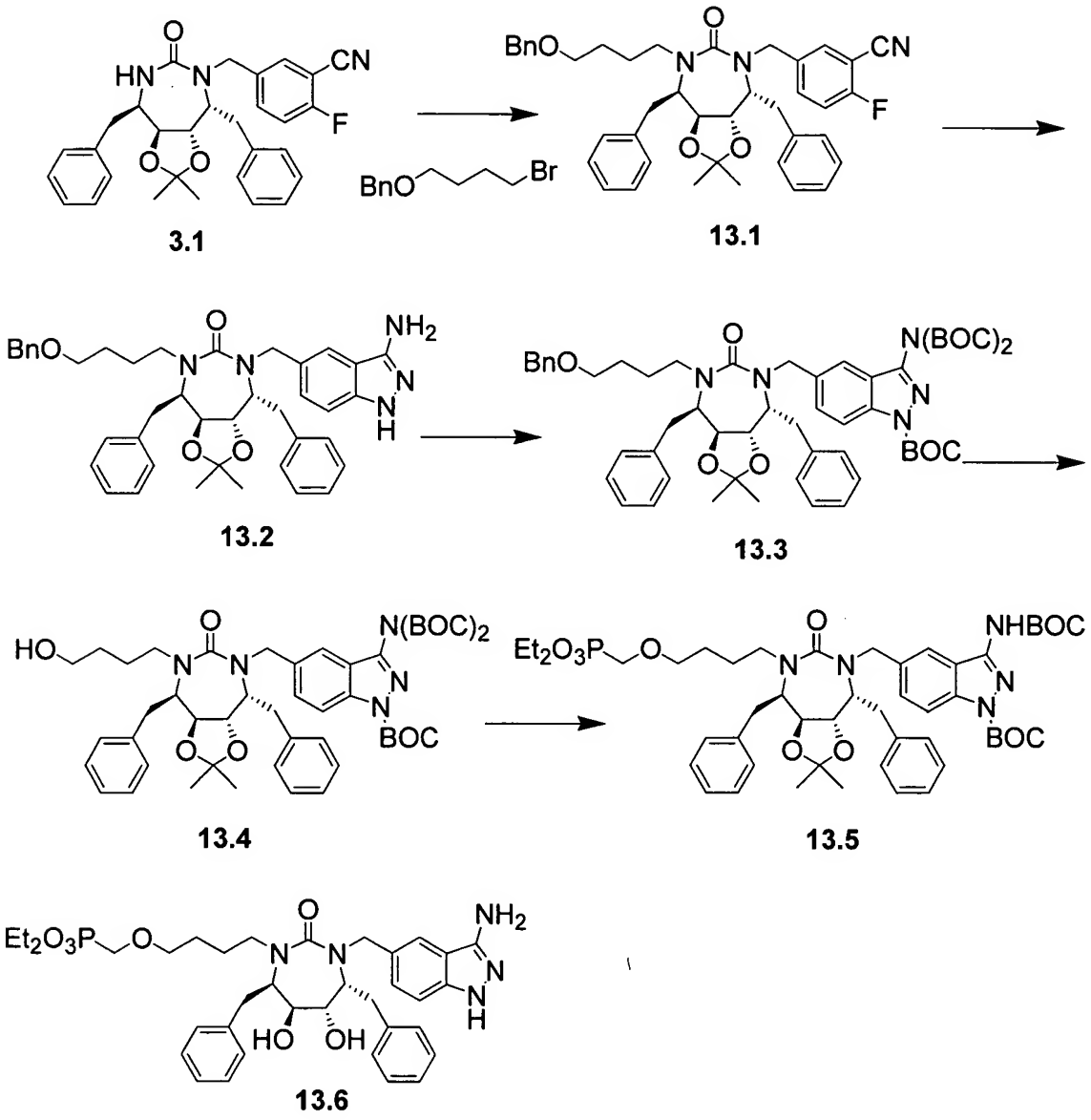
Scheme 12



Butyl lactate phosphonate 12.2: A pyridine solution (0.3 mL) of **11.9** (27 mg, 22 μmol), butyl lactate (31 mg, 265 μmol), and DCC (28 mg, 132 μmol) was stirred at room temperature for 5 min, then reacted at 70°C for 1.5 h. The reaction mixture was concentrated under reduced pressure, partitioned between methylene chloride and saturated NaCl solution, and purified by preparative TLC to give **12.1** (12 mg). A methylene chloride solution (0.8 mL) of **12.1** (12 mg) was treated with TFA (0.2 mL) for 4 h, concentrate. The residue was purified by preparative TLC to give **12.2** (3 mg, 16 %). NMR ($CDCl_3$ + ~10 %CD_3OD): δ 6.8-7.4 (m, 18H), 6.4-6.6 (m), 4.9-5.05 (m, 1H), 4.75 (d, 1H), 4.1-4.2 (m, 2H). 3.5-4.0 (m, 10H), 3.1-3.25 (m, 2H),

2.2-2.35 (m, 1H), 1.8-1.9 (m, 1H), 1.4 & 1.8 (m, 7H), 1.22 (t, 3H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 19.69 & 17.86 ppm.

Scheme 13



Benzyl ether 13.1: A DMF solution (5 mL) of **3.1** (1 g, 2 mmol) was treated with NaH (0.24 g of 60% oil dispersion, 6 mmol) for 30 min, followed by the addition of sodium iodide (0.3 g, 2 mmol),and benzoxybutyl bromide (0.58 g, 2.4 mmol). After the reaction for 5 h at room temperature, the reaction mixture was partitioned between methylene chloride and saturated NaCl, dried, and purified to give **13.1** (0.58 g, 44 %).

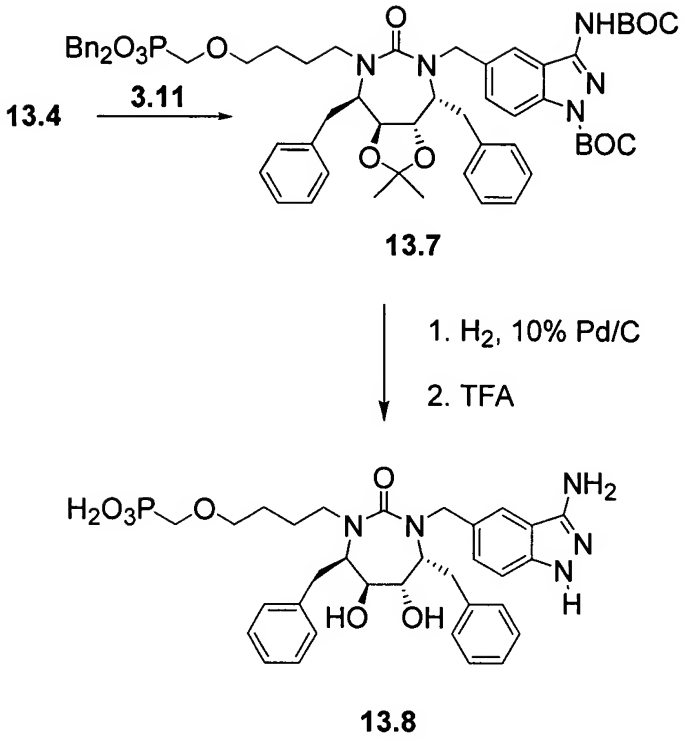
Aminoindazole 13.2: A n-butanol solution (10 mL) of **11.1** (0.58 g, 0.87 mmol) and hydrazine hydrate (0.88 g, 17.5 mmol) was heated at reflux for 4 h. The reaction mixture was concentrated under reduced pressure to give crude **13.2** (0.56 g).

Tri-BOC-aminoindazole 13.3: A methylene chloride solution (10 mL) of **13.2** (0.55 g, 0.82 mmol), DIPEA (0.42 g, 3.2 mmol), $(BOC)_2O$ (0.71 g, 3.2 mmol), and DMAP (0.3 g, 2.4 mmol) was stirred for 4 h at room temperature, partitioned between methylene chloride and 5% citric acid solution, dried, purified by silica gel chromatography to give **13.3** (0.56 g, 71 %, 2 steps).

3-Hydroxybutyl cyclic urea 13.4: An ethyl acetate/methanol solution (30 mL/5 mL) of **11.3** (0.55 g, 0.56 mmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (0.2 g) for 3 h. The catalyst was removed by filtration. The filtrate was concentrated under reduced pressure to afford crude **13.4** (0.5 g, 98 %).

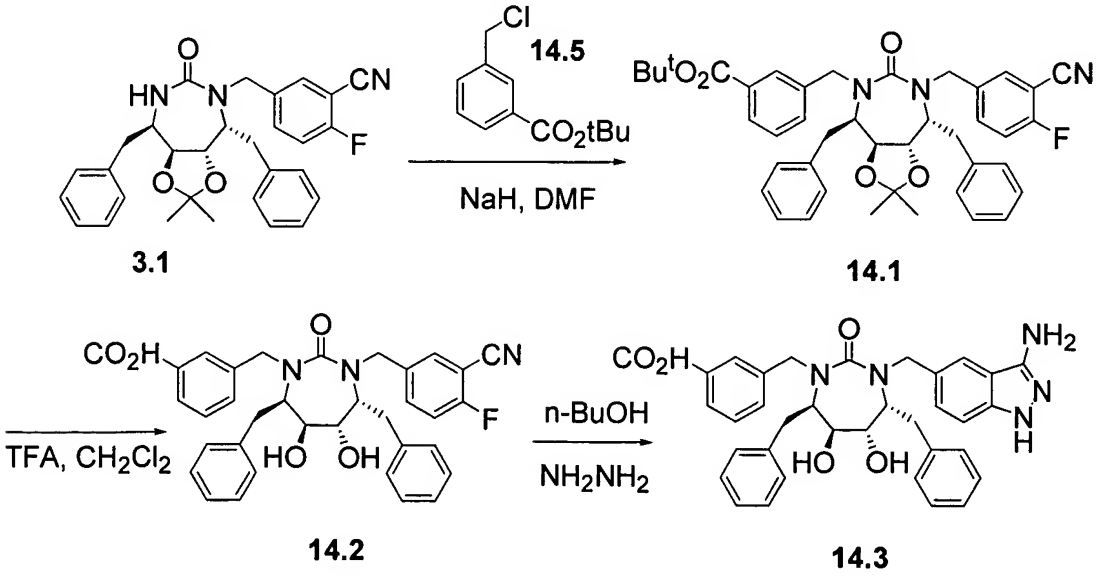
Diethyl phosphonate 13.6: A THF solution (1 mL) of **13.4** (5 mg, 56 μmol) and triflate diethyl phosphonate **5.3** (30 mg, 100 μmol) was cooled to –3°C, followed by addition of n-BuLi (80 μl of 2.5 M hexane solution, 200 μmol). After 2 h reaction, the reaction mixture was partitioned between methylene chloride and saturated NaCl solution, concentrated under reduced pressure to give crude **13.5**. The residue was dissolved in methylene chloride (0.8 mL) and treated with TFA (0.2 mL) for 4 h. concentrated under reduced pressure, and purified by HPLC to give **13.6** (8 mg, 21%). NMR ($CDCl_3$): δ 7.1-7.4 (m, 11H), 7.0-7.1 (m, 2H) 4.81 (d, 1H), 4.1-4.25 (m, 4H). 3.85-3.95 (m, 1H), 3.4-3.8 (m, 7H), 3.3-3.4 (m, 1H), 2.8 -3.25 (m, 5H), 2.0-2.15 (m, 1H), 1.3-1.85 (m, 10H). P NMR ($CDCl_3$): 21.45 ppm.

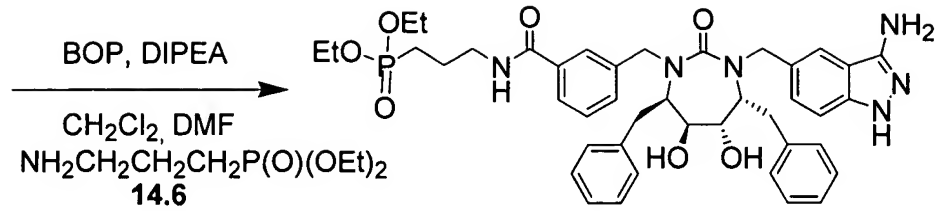
Scheme 13a

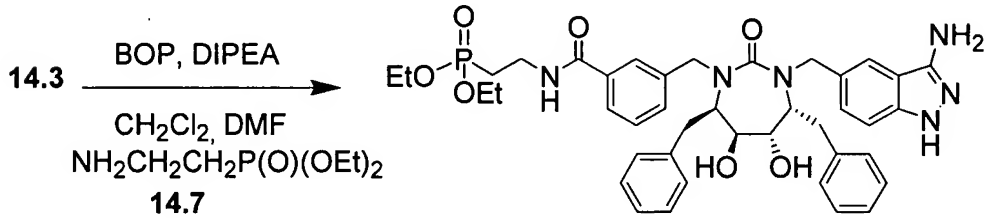


Phosphonic diacid 13.8: Compound **13.8** (4.5 mg) was prepared from **13.4** as described above for the preparation of **11.7** from **11.4** (Scheme 11). NMR (CD_3OD): δ 7.41 (s, 1H), 7.1-7.4 (m, 10H), 6.9-7.0 (m, 2H) 4.75 (d, 1H), 3.8-4.0 (m, 1H). 3.4-3.8 (m, 8H), 2.8 -3.25 (m, 5H), 2.1-2.25 (m, 1H), 1.6-1.85 (m, 4H). MS: 638 (M + 1).

Scheme 14







t-Butyl ester 14.1: A DMF solution (3 mL) of **3.1** (0.5 g, 1 mmol) was treated with NaH (80 mg of 60% oil dispersion, 2 mmol) for 10 min, followed by the addition of **14.5** (0.25 g, 1.1 mmol). After the reaction for 1 h at room temperature, the reaction mixture was partitioned between methylene chloride and saturated NaCl, dried, and purified to give **14.1** (0.4 g, 59%).

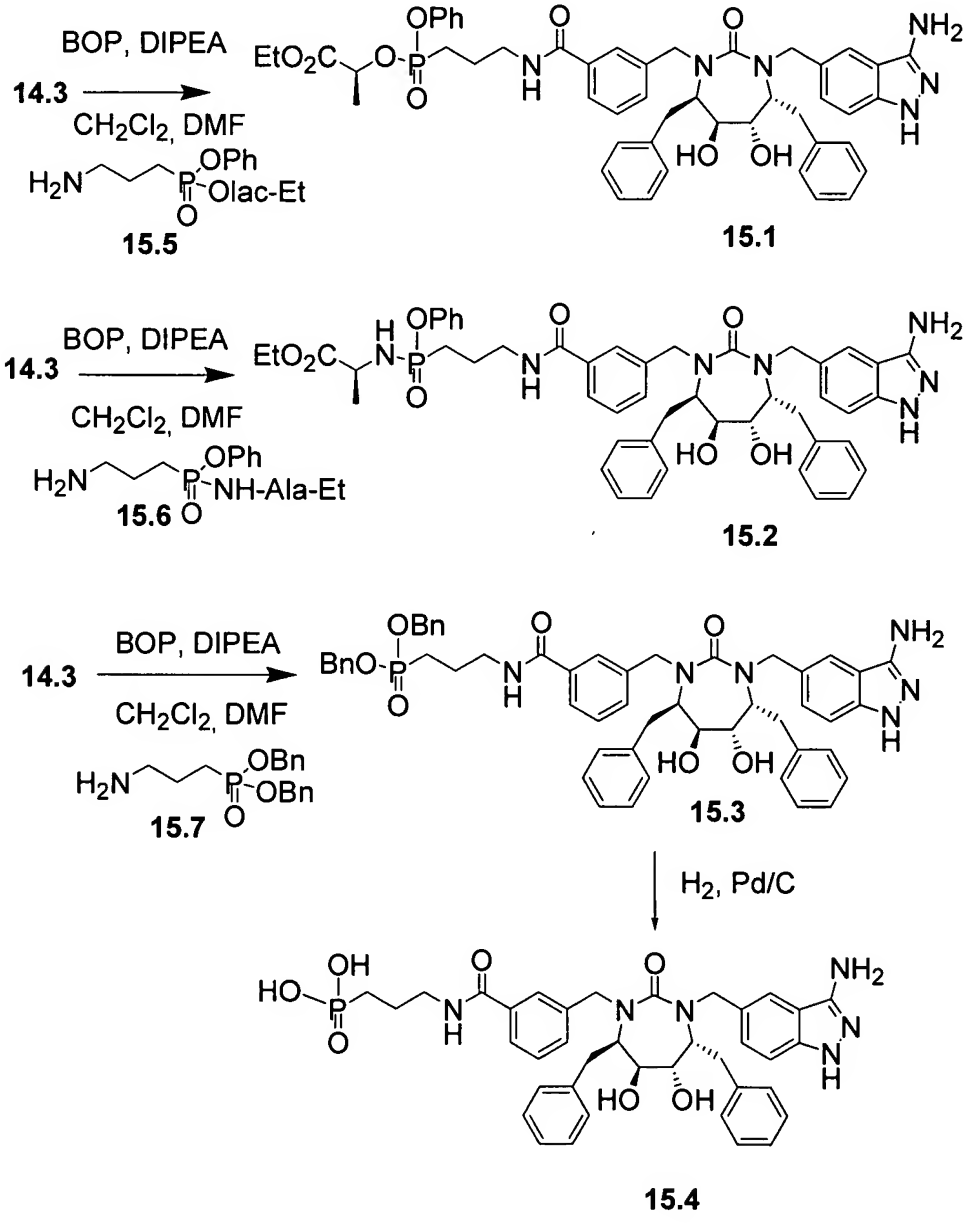
Aminoindazole derivative 14.3: A methylene chloride solution (5 mL) of **14.1** (0.4 g, 0.58 mmol) was treated with TFA (1 mL) at room temperature for 1.5 h, and then concentrated under reduced pressure to give crude **14.2**. The crude **14.2** was dissolved in n-BuOH (5 mL) and reacted with hydrazine hydrate (0.58 g, 11.6 mmol) at reflux for 5 h. The reaction mixture was concentrated under reduced pressure and purified by silica gel chromatography to give the desired product **14.3** (0.37 g, quantitative yield).

Diethylphosphonate ester 14.4: A methylene chloride solution (3 mL) of **14.3** (23 mg, 38 μmol) was reacted with aminopropyl-diethylphosphonate **14.6** (58 mg, 190 μmol), DIPEA (50 mg, 380 μmol), and ByBOP (21 mg, 48 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by preparative TLC to give **14.4** (9 mg, 34 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.87 (t, 1H), 7.61 (b, 1H), 7.51 (s, 1H), 7.14-7.2 (m, 10 H), 6.93-7.0 (m, 4H), 4.79 (d, 2H), 3.99-4.04 (m, 4H), 3.38-3.65 (m, 6H), 2.60-3.2 (m, 6 H), 1.70-1.87 (m, 4H), 1.25 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 32.7 ppm.

Diethylphosphonate ester 14.5: A methylene chloride solution (2 mL) of **14.3** (13 mg, 21 μmol) was reacted with aminoethyl-diethylphosphonate oxalate **14.7** (23mg, 85 μmol), DIPEA (22 mg, 170 μmol), and ByBOP (12 mg, 25 μmol) at room temperature for 2 h, and then

concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by preparative TLC to give 14.5 (5mg, 30%). Ms: 783 (M + 1). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.88 (b, 1H), 7.58 (b, 1H), 7.49 (s, 1H), 7.14-7.2 (m, 10 H), 6.90-7.0 (m, 4H), 4.75 (d, 2H), 3.90-4.04 (m, 4H), 2.50-3.3 (m, 6 H), 1.97-2.08 (m, 2H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 30.12 ppm.

Scheme 15



Monophenol-ethyl lactate phosphonate prodrug 15.1: A methylene chloride/DMF solution (2 mL/0.5 mL) of **14.3** (30 mg, 49 μmol) was reacted with aminopropyl-phenol-ethyl lactate phosphonate **15.5** (100 mg, 233 μmol), DIPEA (64 mg, 495 μmol), and BOP reagent (45 mg, 100 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by silica gel chromatography to give **15.1** (28 mg, 64 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.83 (b, 1H), 7.59 (b, 1H), 7.51 (s, 1H), 7.14-7.2 (m, 11 H), 6.90-7.0 (m, 4H), 4.75-4.87 (d + q, 3H), 4.10 (q, 2H), 3.3-3.61 (m, 6H), 2.60-3.2 (m, 6H), 1.92-2.12 (m, 4H), 1.30 (d, 3H), 1.18 (t, 3H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 30.71 ppm. MS: 903 (M + 1).

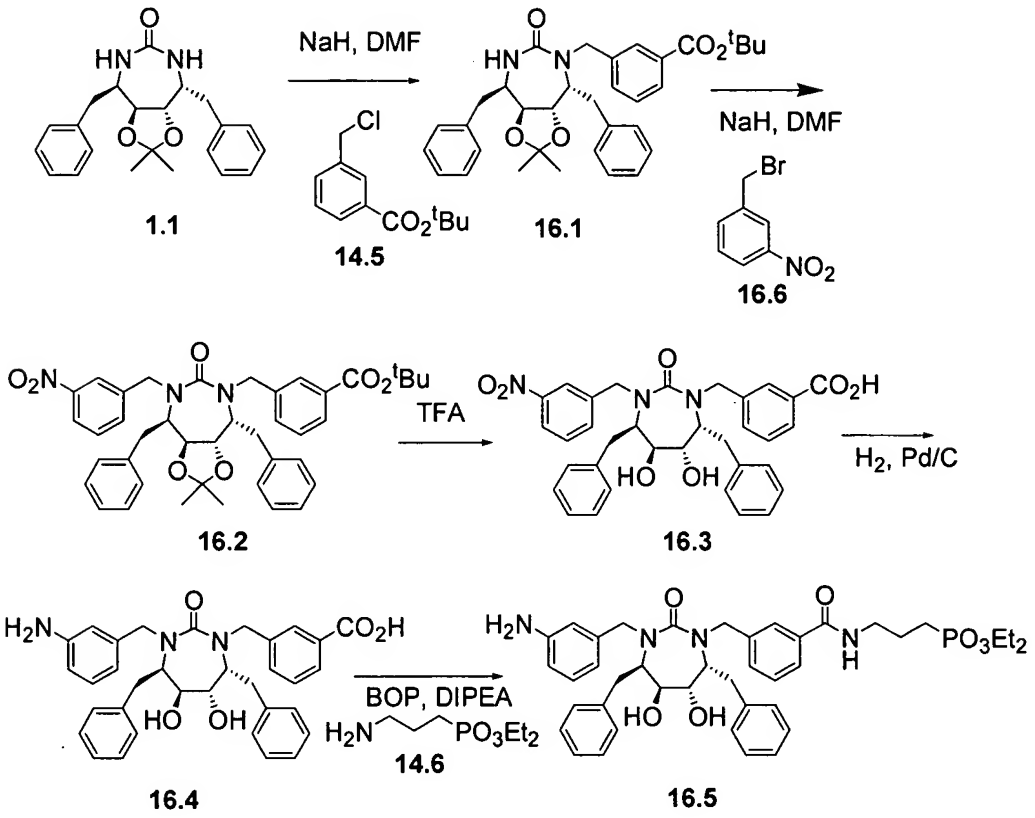
Phenol-ethyl alanine phosphonate prodrug 15.2: A methylene chloride/DMF solution (2 mL/0.5 mL) of **14.3** (30 mg, 49 μmol) was reacted with aminopropyl-phenol-ethyl alanine phosphonate **15.6** (80 mg TFA salt, 186 μmol), DIPEA (64 mg, 500 μmol), and BOP reagent (45 mg, 100 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by preparative TLC to give **15.2** (12 mg, 27 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.91 (b, 1H), 7.61 (b, 1H), 7.52 (s, 1H), 7.14-7.2 (m, 11 H), 6.90-7.0 (m, 4H), 4.75 (d, 2H), 3.82-4.1 (2q, 3H), 3.4-3.65 (m, 6H), 2.60-3.15 (m, 6H), 1.8-2.0 (m, 4H), 1.3 (d, 3H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 32.98 & 33.38 ppm. MS: 902 (M + 1).

Dibenzyl phosphonate 15.3: A methylene chloride/DMF solution (2 mL/0.5 mL) of **14.3** (30 mg, 49 μmol) was reacted with aminopropyl dibenzyl phosphonate **15.7** (86 mg TFA salt, 200 μmol), DIPEA (64 mg, 500 μmol), and BOP reagent (45 mg, 100 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by preparative TLC to give **15.3** (20 mg, 44%). NMR ($CDCl_3$ + ~5%CD_3O): δ 7.50-7.58 (m, 2H), 7.14-7.3 (m, 21 H), 6.90-7.0 (m, 4H), 4.7-5.1 (m, 6H), 3.6-3.8 (m, 4H), 3.3-3.55 (m, 2H), 2.60-3.15 (m, 6H), 1.8-2.0 (m, 4H). P NMR ($CDCl_3$ + ~5 %CD_3OD): 33.7 ppm. MS: 907 (M + 1).

Phosphonic diacid 15.4: An ethanol solution (5 mL) of **15.3** (17 mg, 18.7 μmol) was hydrogenated at 1 atm in the presence of 10 % Pd/C for 4 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired product **15.4** (12 mg, 85%). NMR (CD_3O + 20%$CDCl_3$): δ 7.88 (b, 1H), 7.59 (b, 1H), 7.6 (s, 1H), 7.1-

7.25 (m, 10 H), 6.90-7.1 (m, 4H), 4.8 (d, 2H + water peak), 3.6-3.8 (m, 4H), 3.4-3.5 (m, 2H), 1.85-2.0 (m, 4H).

Scheme 16



Monobenzyl derivative 16.1: A DMF solution (4 mL) of **1.1** (0.8 g, 2.2 mmol) was treated with NaH (0.18 g of 60% oil dispersion, 4.4 mmol) for 10 min at room temperature followed by the addition of 14.5 (0.5 g, 2.2 mmol). The resulting solution was reacted at room temperature for 2 h, worked up, and then purified to afford **16.1** (0.48 g, 40%).

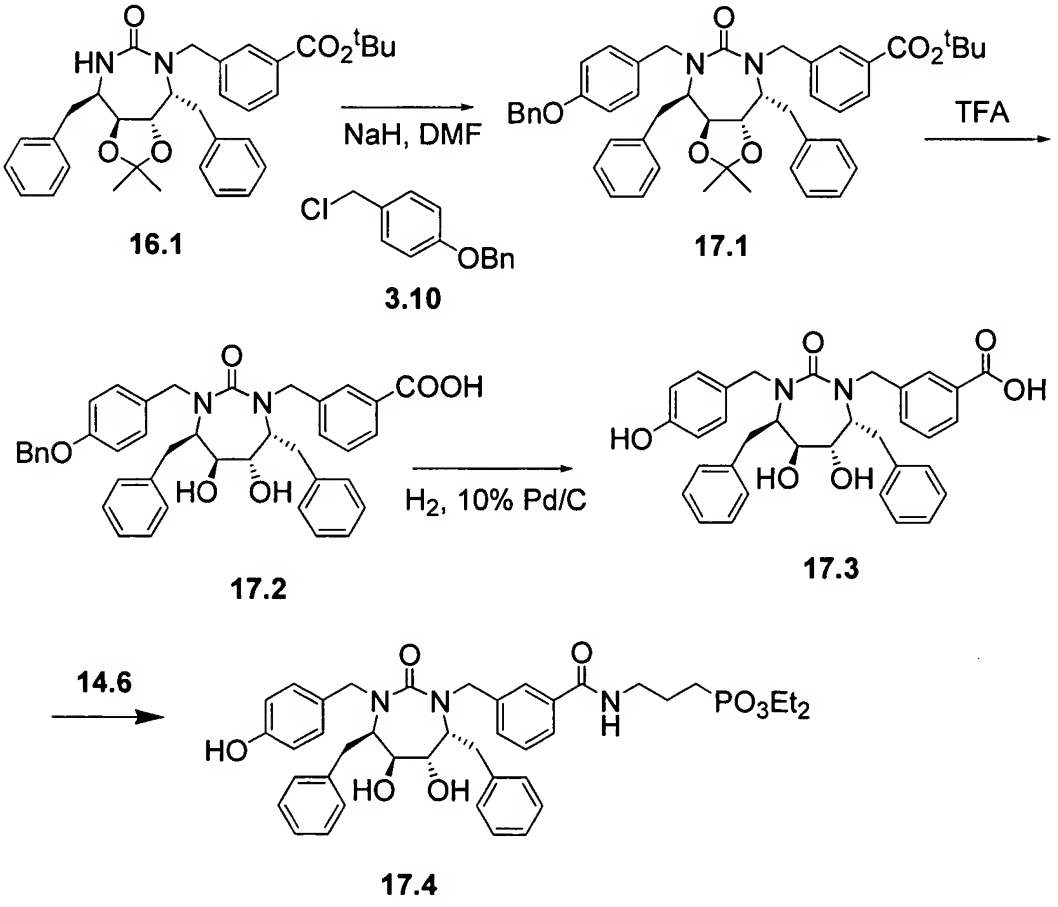
3-Nitrobenzyl cyclic urea derivative 16.2: A DMF solution (0.5 mL) of **16.1** (65 mg, 117 μmol) was treated with NaH (15 mg of 60% oil dispersion, 375 μmol) for 10 min at room temperature, followed by the addition of 3-nitrobenzyl bromide (33 mg, 152 μmol). The resulting solution was reacted at room temperature for 1 h, worked up, and purified by preparative TLC to afford **16.2** (66 mg, 82%).

Diol 16.3: A methylene chloride solution (2 mL) of **16.2** (46 mg, 61 μmol) was treated with TFA (0.4 mL) for 2 h at room temperature, and then concentrated under reduced pressure to afford **16.3**. This material was used without further purification.

3-Aminobenzyl cyclic urea 16.4: An ethyl acetate/ethanol (5 mL/1 mL) solution of **16.3** (crude) was hydrogenated at 1 atm in the presence of 10% Pd/C for 2 h. The catalyst was removed by filtration. The filtrate was concentrated under reduced pressure, and purified by preparative TLC to afford **16.4** (26 mg, 70%, 2 steps).

Diethyl phosphonate 16.5: A methylene chloride/DMF solution (2 mL/0.5 mL) of **16.4** (24 mg, 42 μmol) was reacted with aminopropyl-diethylphosphonate ester TFA salt **14.6** (39 mg, 127 μmol), DIPEA (27 mg, 210 μmol), and BOP reagent (28 mg, 63 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was purified by preparative TLC to give **16.5** (20.7 mg, 63 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.62 (b, 1H), 7.51 (s, 1H), 7.0-7.35 (m, 12 H), 6.95 (d, 2H), 6.85 (d, 2H), 4.6-4.71 (2d, 2H), 3.95-4.1 (m, 4H). 3.3-3.55 (m, 3H), 2.60-2.8 (m, 2H), 2.95-.3. 15 (m, 4 H), 1.85-2.0 (m, 4H), 1.25 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 32.65 ppm.

Scheme 17

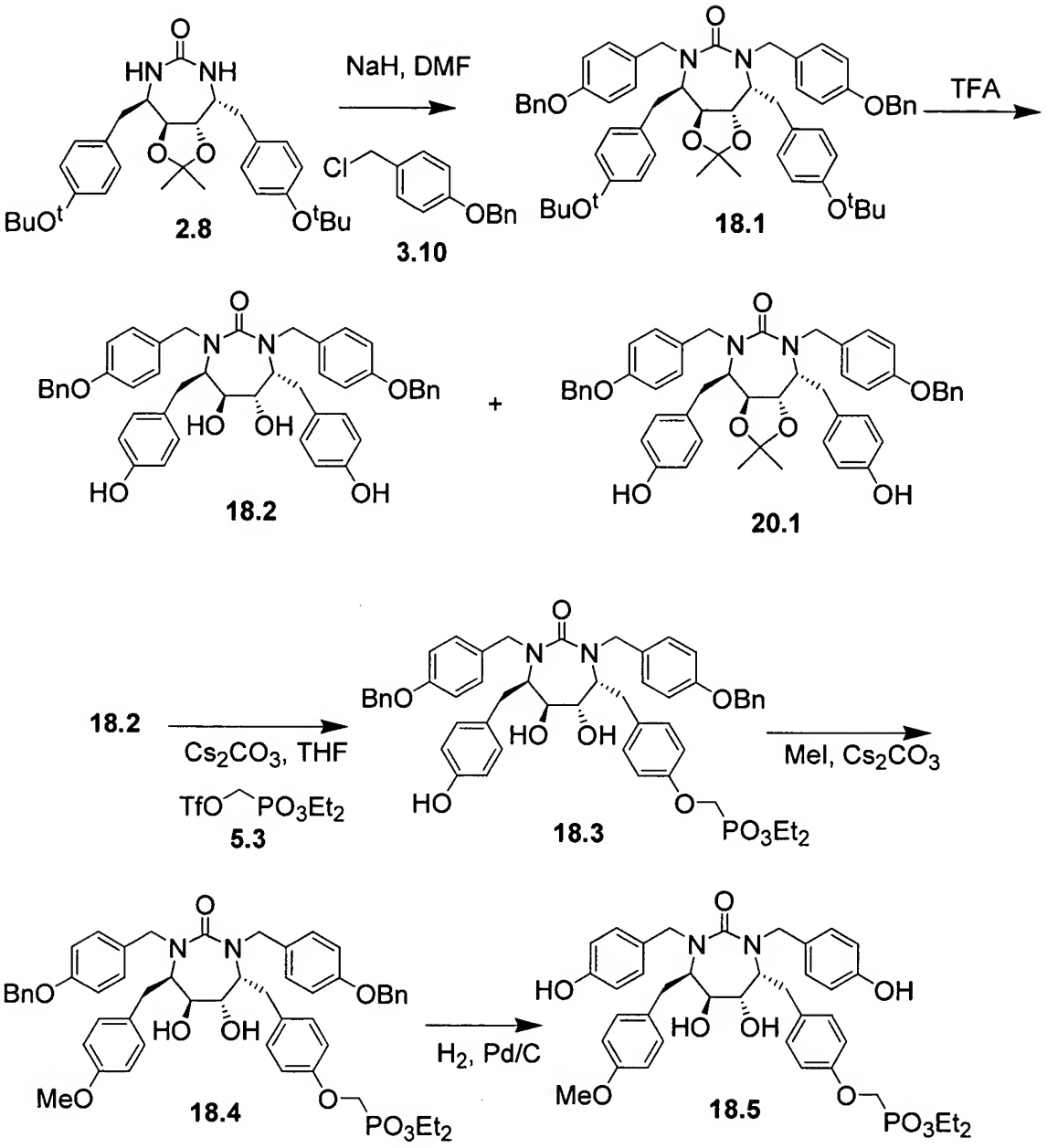


p-Benzoxybenzyl cyclic urea derivative 17.1: A DMF solution (0.5 mL) of **16.1** (65 mg, 117 μmol) was treated with NaH (15 mg of 60% oil dispersion, 375 μmol) for 10 min at room temperature, followed by the addition of 4-benzoxy benzyl chloride **3.10** (35 mg, μmol). The resulting solution was stirred for 2 h at room temperature. The reaction mixture was concentrated under reduced pressure, purified by preparative TLC to generate **17.1** (62 mg, 70%).

Diethyl phosphonate 17.3: A methylene chloride solution (2 mL) of **17.1** (46 mg, 61 μmol) was treated with TFA (0.4 mL) for 2 h at room temperature, and then concentrated under reduced pressure to give crude **17.2**. An ethyl acetate/ethanol solution (3 mL/2 mL) of the crude **17.2** was then hydrogenated at 1 atm in the presence of 10% Pd/C (10 mg) for 5 h at room temperature. The catalyst was removed by filtration. The filtrate was concentrated under reduced pressure to afford **17.3** (crude).

Diethyl phosphonate cyclic urea 17.4: A methylene chloride/DMF solution (2 mL/0.5 mL) of **17.3** (25 mg, 42 μmol) was reacted with aminopropyl-diethylphosphonate ester TFA salt **14.6** (40 mg, 127 μmol), DIPEA (27 mg, 210 μmol), and BOP reagent (28 mg, 63 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was purified by preparative TLC to give **17.4** (14.6 mg, 44 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.82 (t), 7.62 (d, 1H), 7.51 (s, 1H), 7.05-7.35 (m, 10 H), 6.8-6.95 (2d, 4H), 6.85 (d, 2H), 4.8 (d, 1H), 4.65 (d, 1H), 3.95-4.1 (m, 4H). 3.4-3.75 (m, 6H), 2.60-3.2 (m), 1.85-2.0 (m, 4H), 1.25 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 32.72 ppm.

Scheme 18



Dibenzyl derivative 18.1: A DMF solution (3 mL) of compound **2.8** (0.4 g, 0.78 mmol) was reacted with 60%NaH (0.13 g, 1.96 mmol), 4-benzoxy benzylchloride **3.10** (0.46 g, 1.96 mmol) and sodium iodide (60 mg, 0.39 mmol) at room temperature for 4 h. The reaction mixture was partitioned between methylene chloride and saturated $NaHCO_3$ solution. The organic phase was isolated, dried over Na_2SO_4, concentrated under reduced pressure, and purified by silica gel chromatography to give the desired product **18.1** (0.57 g, 81%).

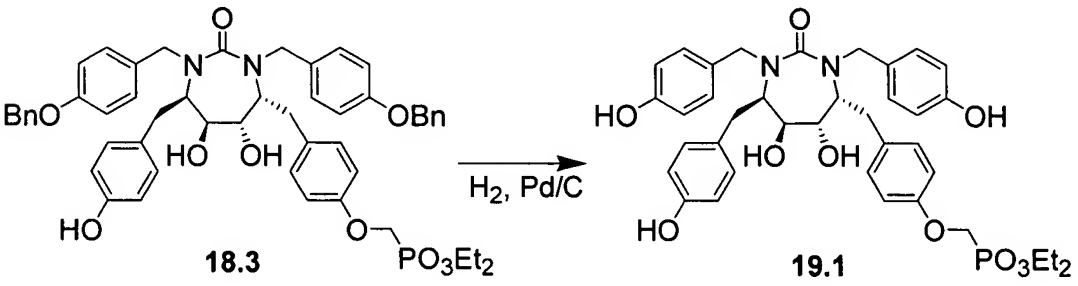
Diol derivative 18.2 and diphenol derivative 20.1: A methylene chloride solution (4 mL) of **18.1** (0.57 g, 0.63 mmol) was treated with TFA (1 mL) at room temperature for 20 min, concentrated under reduced pressure, and purified by silica gel chromatography to give diol derivative **18.2** (133 mg, 28 %) and diphenol derivative **20.1** (288 mg. 57.6%).

Monophosphonate derivative 18.3: A THF solution (10 mL) of **18.2** (130 mg, 0.17 mmol) was stirred with cesium carbonate (70 mg, 0.21 mmol) and diethylphosphonate triflate **5.3** (52 mg, , 0.17 mmol) at room temperature for 4 h.. The reaction mixture was concentrated under reduced pressure and purified to give **18.3** (64 mg, 41 %), and recovered **18.2** (25 mg, 19%).

Methoxy derivative 18.4: A THF solution (2 mL) of **18.3** (28 mg, 25 μmol) was treated with cesium carbonate (25 mg, 76 μmol) and iodomethane (10 eq. Excess) at room temperature for 5 h. The reaction mixture was concentrated under reduced pressure and partitioned between methylene chloride and saturated $NaHCO_3$. The organic phase was separated, concentrated under reduced pressure and the residue purified by preparative TLC to afford **18.4** (22 mg, 78%).

Diethylphosphonate 18.5: An ethyl acetate/ethanol (2 mL/2 mL) solution of **18.4** (22 mg, 24 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C for 3 h. The catalyst was removed by filtration, the filtrate was concentrated under reduced pressure to give the desired product **18.5** (18 mg, quantitative). NMR ($CDCl_3$ + ~10 %CD_3O): δ 6.7-7.0 (m, 12 H), 6.62-6.69 (m, 4H), 4.65 (d, 1H), 4.50 (d, 1H), 4.18-4.3 (m, 6H). 3.75 (s, 3H), 3.3-3.4 (m, 4H), 2.8-3.0 (m, 6H), 1.30 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 20.16 ppm.

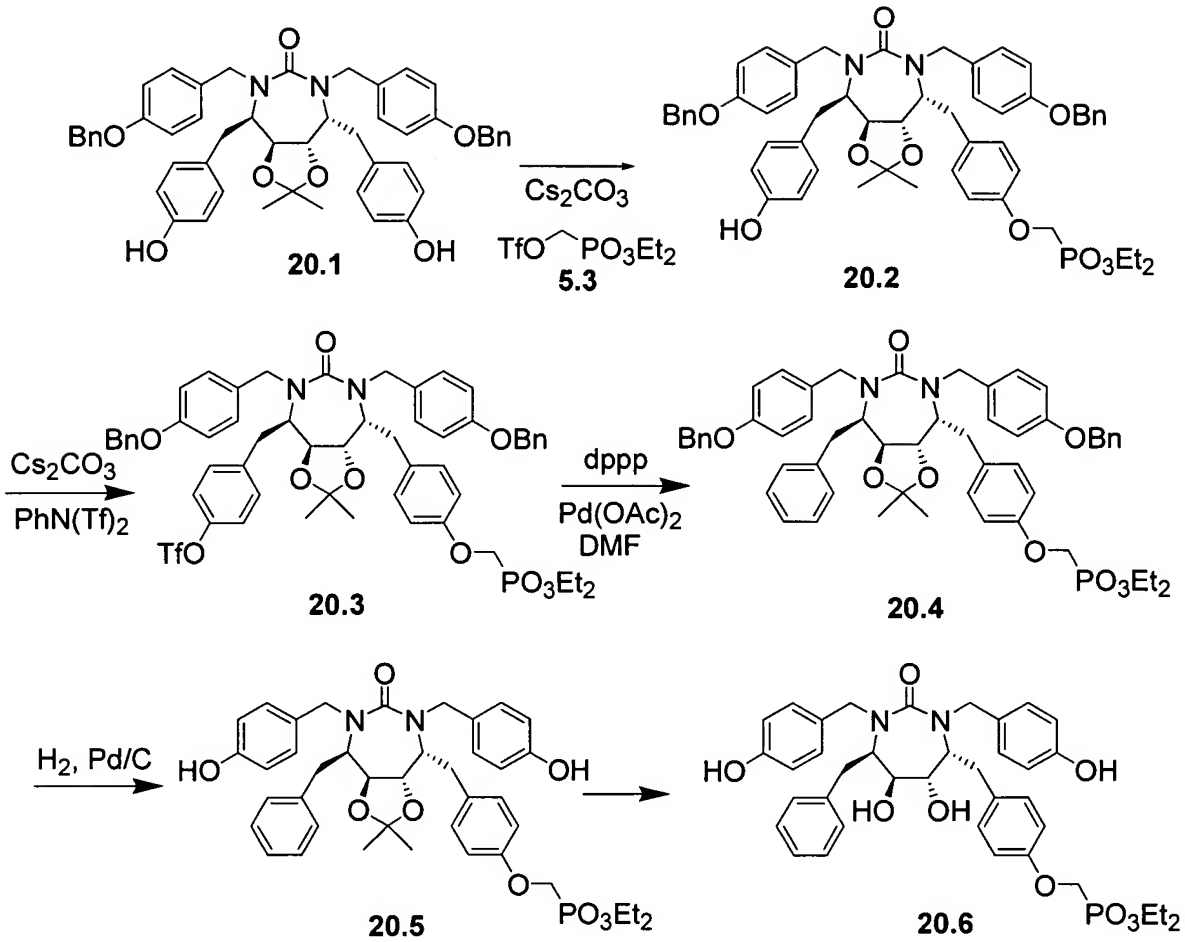
Scheme 19



Diethyl phosphonate 19.1: An ethyl acetate/ethanol (2 mL/1 mL) solution of **18.3** (14 mg, 15.5 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (5 mg) for 3 h. The catalyst was then removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired product **19.1** (10 mg, 90%). NMR ($CDCl_3$ + ~15 %CD_3O): δ 6.6-7.0 (m, 16

H), 4.5-4.65 (2d, 2H), 4.1-4.3 (m, 6H). 2.7-3.0 (m, 6H), 1.29 (t, 6H). P NMR ($CDCl_3$ + ~15 %CD_3OD): 20.12 ppm.

Scheme 20



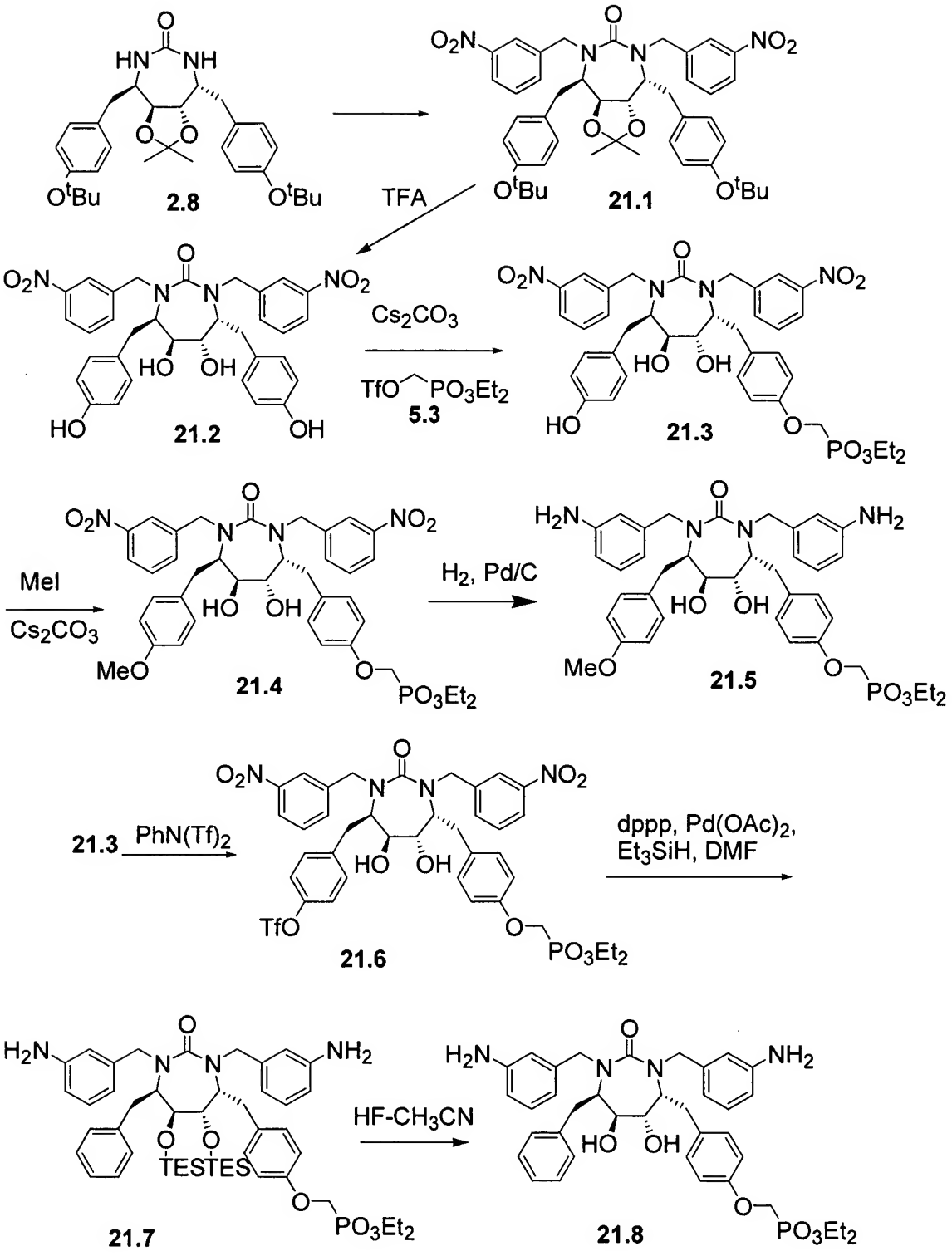
Monophosphonate 20.2: A THF solution (8 mL) of **20.1** (280 mg, 0.36 mmol) was stirred with cesium carbonate (140 mg, 0.43 mmol) and diethylphosphonate triflate **5.3** (110 mg, 0.36 mmol) at room temperature for 4 h.. The reaction mixture was concentrated under reduced pressure and purified to give **20.2** (130mg, 39%), and recovered **20.1** (76 mg, 27%).

Triflate derivative 20.3: A THF solution (6 mL) of **20.2** (130 mg, 0.13 mmol) was stirred with cesium carbonate (67 mg, 0.21 mmol) and N-phenyltrifluoromethane-sulfonimide (60mg, 0.17 mmol) at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and purified to give **20.3** (125 mg, 84%).

Benzyl ether 20.4: To a DMF solution (2 mL) of $Pd(OAc)_2$ (60 mg, 267 μmol), and dppp (105 mg. 254 μmol) was added **20.3** (120 mg, 111 μmol) under nitrogen, followed by the addition of triethylsilane (0.3 mL). The resulting solution was stirred at room temperature for 4 h, then concentrated under reduced pressure. The residue was purified by silica gel chromatography to afford **20.4** (94 mg, 92%).

Diethyl phosphonate 20.6: An ethyl acetate/ethanol (2 mL/2 mL) solution of **20.4** (28 mg, 30 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (5 mg) for 3 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired product **20.5**. The crude product **20. 5** was redissolved in methylene chloride (2 mL) and treated with TFA (0.4 mL) and a drop of water. After 1 h stirring at room temperature, the reaction mixture was concentrated under reduced pressure, and purified by preparative TLC plate to give **20.6** (18 mg, 85 %, 2 steps). δ 6.6-7.3 (m, 17 H), 4.65 (d, 1H), 4.58 (d, 1H), 4.18-4.3 (m, 6H), 3.3-3.5 (m, 4H), 2.8-3.1 (m), 1.34 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 20.16 ppm. MS: 705 (M + 1).

Scheme 21



Bis-(3-nitrobenzyl) derivative 21.1: A DMF solution (2 mL) of compound **2.8** (0.3 g, 0.59 mmol) was reacted with 60%NaH (0.07 g, 1.76 mmol), 3-nitrobenzyl bromide (0.38 g, 1.76

mmol) and sodium iodide (60 mg, 0.39 mmol) at room temperature for 3 h. The reaction mixture was partitioned between methylene chloride and saturated $NaHCO_3$ solution. The organic phase was isolated, dried over Na_2SO_4, concentrated under reduced pressure, and purified by silica gel chromatography to give the desired product **21.1** (0.37 g, 82%).

Diphenol derivative 21.2: A methylene chloride solution (4 mL) of **21.1** (0.37 g, 0.47 mmol) was treated with TFA (1 mL) at room temperature for 3 h, and then concentrated under reduced pressure, and azeotroped with CH_3CN twice to give diphenol derivative **21.2** (0.3 g, quantitative).

Monophosphonate derivative 21.3: A THF solution (8 mL) of **18.2** (0.28g, 0.44 mmol) was stirred with cesium carbonate (0.17 g, 0.53 mmol) and diethylphosphonate triflate **5.3** (0.14 g, 0.44 mmol) at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and purified to give **21.3** (120 mg, 35%), and recovered **21.2** (150 mg, 53%).

Methoxy derivative 21.4: A THF solution (2 mL) of **21.3** (9 mg, 11 μmol) was treated with cesium carbonate (15 mg, 46 μmol) and iodomethane (10 eq. Excess) at room temperature for 6 h. The reaction mixture was concentrated under reduced pressure and partitioned between methylene chloride and saturated $NaHCO_3$. The organic phase was separated, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by preparative TLC to afford **21.4** (9 mg).

Diethylphosphonate 21.5: A ethyl acetate/ethanol (2 mL/0.5 mL) solution of **21.4** (9 mg, 11 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C for 4 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired product 21.5 (4.3 mg, 49%, 2 steps). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.0-7.10 (m, 6 H), 6.8-6.95 (m, 4H), 6.5-6.6 (m, 4H), 6.4-6.45 (m, 2H), 4.72 (d, 2H), 4.18-4.3 (m, 6H). 3.72 (s, 3H), 3.4-3.5 (m, 4H), 2.8-3.0 (m, 6H), 1.34 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 19.93 ppm.

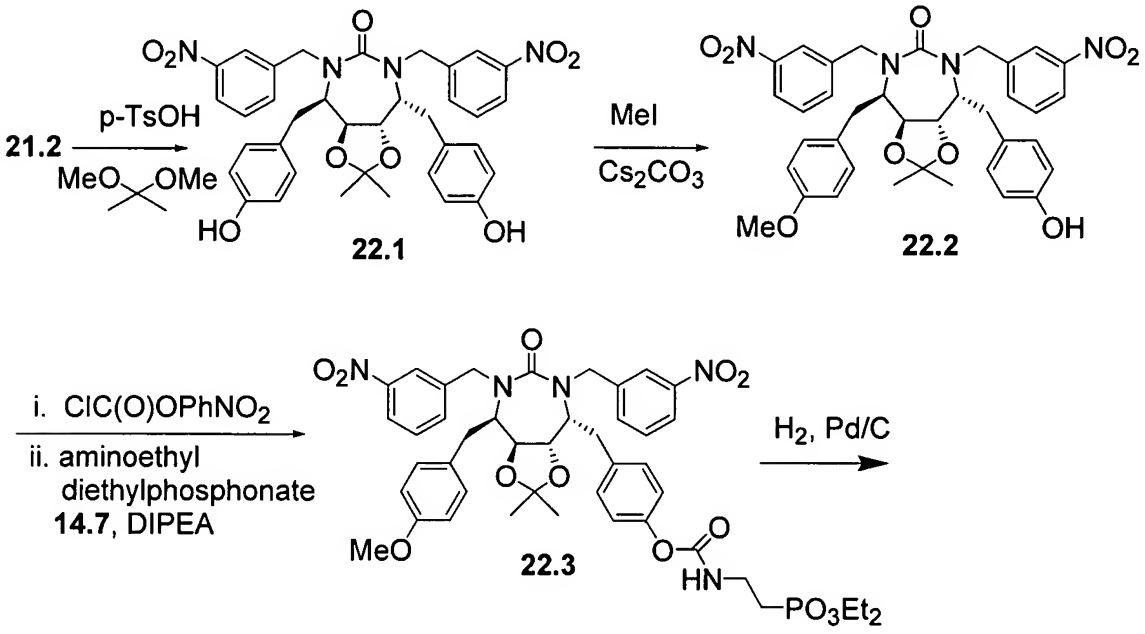
Triflate 21.6: A THF solution (6 mL) of **21.3** (0.1g, 0.14 mmol), cesium carbonate (0.07 g, 0.21 mmol), and N-phenyltrifluoromethane-sulfonimide (60mg, 0.17 mmol) was stirred at room temperature for 4 h, and then concentrated under reduced pressure, and worked up. The residue was purified by silica gel chromatography to give **21.6** (116 mg, 90%).

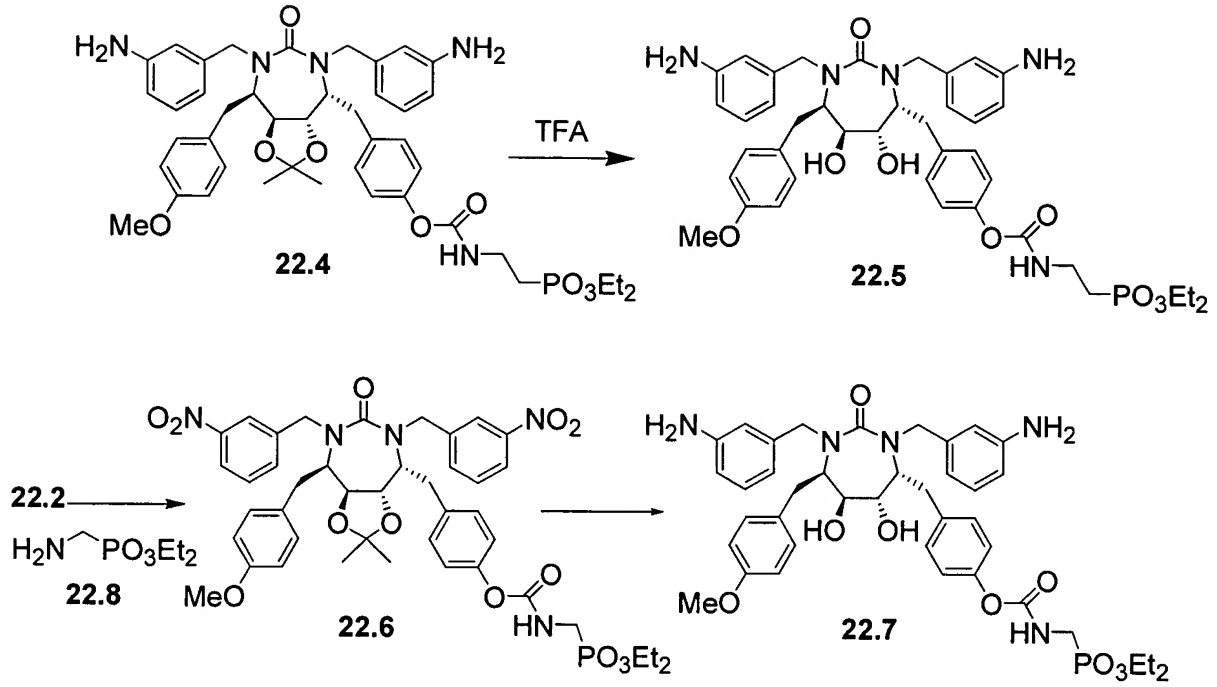
Diamine 21.7: A DMF solution (2 mL) of **21.6** (116 mg, 127 μmol), dppp (60 mg, 145 μmol), and $Pd(OAc)_2$ (30 mg, 134 μmol) was stirred under nitrogen, followed by addition of

triethylsilane (0.3 mL), and reacted for 4 h at room temperature. The reaction mixture was worked up and purified to give **21.7** (50 mg).

Diethyl phosphonate 21.8: An acetonitrile solution (1 mL) of crude **21.7** (50 mg) was treated with 48% HF (0.1 mL) for 4 h. The reaction mixture was concentrated under reduced pressure, and purified to give **21.8** (10 mg, 11% (2 steps). NMR ($CDCl_3$ + ~10%CD_3O): δ 7.05-7.30 (m, 9 H), 6.8-6.95 (d, 2H), 6.4-6.6 (m, 6H), 4.72 (d, 2H), 4.18-4.3 (m, 6H). 3.4-3.5 (m, 4H), 2.8-3.0 (m, 6H), 1.34 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 19.83 ppm.

Scheme 22





Acetonide 22.1: An acetone/2,2-diemethoxypropane solution (15 mL/5 mL) of compound **21.2** (240 mg, 0.38 mmol) and pyridinium toluenesulfonate (10 mg) was heated at reflux for 30 min. After cooled to room temperature, the reaction mixture was concentrated under reduced pressure. The residue was partitioned between methylene chloride and saturated $NaHCO_3$ aqueous solution, dried, concentrated under reduced pressure and purified to afford **22.1** (225 mg, 88%).

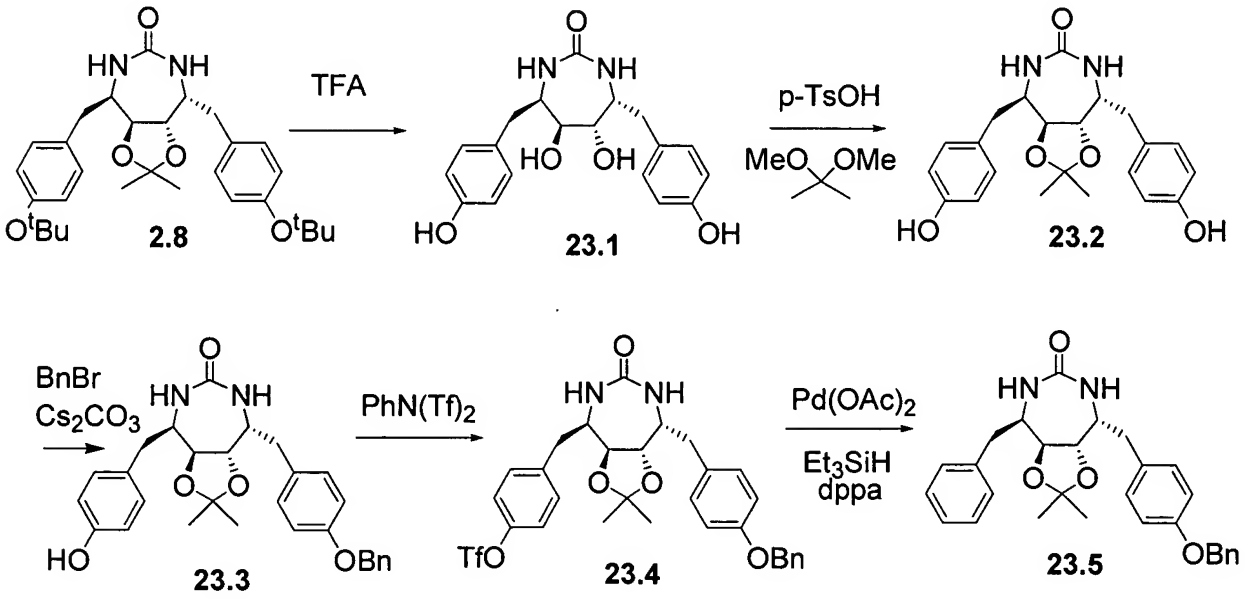
Monomethoxy derivative 22.2: A THF solution (10 mL) of **22.1** (225 mg, 0.33 mmol) was treated with cesium carbonate (160 mg, 0.5 mmol) and iodomethane (52 mg. 0.37 mmol) at room temperature overnight. The reaction mixture was concentrated under reduced pressure, and purified by preparative silica gel column chomatography to afford **22.2** (66 mg, 29%) and recovered starting material **22.1** (25 mg, 11%).

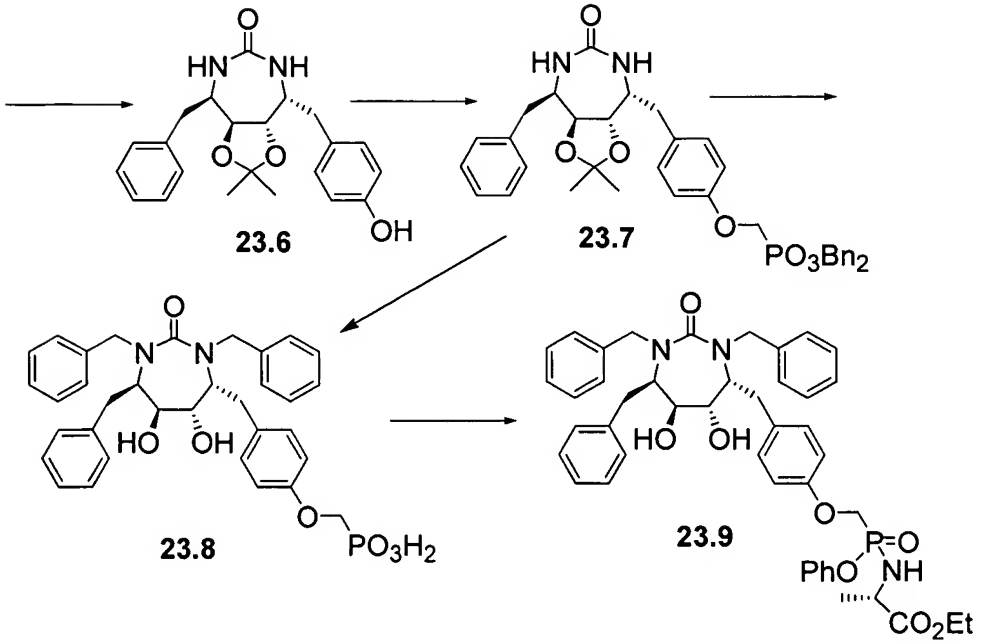
Diethyl phosphonate 22.3: A methylene chloride solution (2 mL) of **22.2** (22 mg, 32 μmol), DIPEA (9 mg, 66 μmol), and p-nitrophenyl chloroformate (8 mg, 40 μmol) was stirred at room temperature for 30 min. The resulting reaction mixture was reacted with DIPEA (10 mg, 77 μmol), and aminoethyl diethylphosphonate **14.7** (12 mg. 45 μmol) at room temperature overnight. The reaction mixture was washed with 5% citric acid solution, saturated $NaHCO_3$, dried, and purified by preparative TLC to afford **22.3** (12 mg, 43%).

Bis(3-aminobenzyl)-diethylphosphonate ester 22.5: An ethyl acetate/t-BuOH (4 mL/2 mL) solution of **22.3** (12 mg, 13 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C 95 mg) at room temperature for 5 h. The catalyst was removed by filtration. The filtrate was concentrated under reduced pressure, and purified by preparative TLC to give **22.4** (8 mg, 72%). A methylene chloride solution (0.5 mL) of **22.4** (8 mg) was treated with TFA (0.1 mL) at room temperature for 1 h., concentrated under reduced pressure, and then azeotroped with CH_3CN twice to afford **22.5** (8.1 mg, 81%). NMR ($CDCl_3$ + ~10 %CD_3OD): δ 7.2 (d, 1H), 6.95-7.15 (m, 6H), 6.75-6.9 (m, 5 H), 4.66 (d, 1H), 4.46 (d, 1H), 4.06-4.15 (m, 4H). 3.75 (s, 3H), 3.6-3.7 (m, 4H), 2.6-3.1 (m, 6H), 2.0-2.1 (m, 2H), 1.30 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 29.53 ppm. MS: 790 (M + 1).

Bis(3-aminobenzyl) diethylphosphonate ester 22.7: Compound **22.7** was prepared from **22.2** (22 mg, 32 μmol) and aminomethyl diethylphosphonate **22.8** as shown above for the preparation of **22.5** from **22.2.** NMR ($CDCl_3$ + ~10 %CD_3OD): δ 7.24 (d, 1H), 6.8-7.12 (m, 11H), 4.66 (d, 1H), 4.45 (d, 1H), 4.06-4.15 (m, 4H). 3.75 (s, 3H), 2.6-3.1 (m, 6H), 1.30 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 22.75 ppm. MS: 776 (M + 1).

Scheme 23





Diol 23.1: To a solution of compound **2.8** (2.98 g, 5.84 mmol) in methylene chloride (14 mL) was added TFA (6 mL). The resulted mixture was stirred at room temperature for 2 h. Methanol (5 mL) and additional TFA (5 mL) were added. The reaction mixture was stirred for additional 4 h and then concentrated under reduced pressure. The residue was washed with hexane/ethyl acetate (1:1) and dried to afford compound **23.1** (1.8 g, 86%) as an off-white solid.

Benzyl ether 23.3: To a solution of compound **23.1** (1.8 g, 5.03 mmol) in DMF (6 mL) and 2,2-dimethoxyl propane (12 mL) was added p-toluenesulfonic acid monohydrate (0.095 g, 0.5 mmol). The resultant mixture was stirred at 65°C for 3 h. The excess 2,2-dimethoxyl propane was slowly distilled. The reaction mixture was cooled to room temperature and charged with THF (50 mL), benzyl bromide (0.8 mL, 6.73 mmol) and cesium carbonate (2.0 g, 6.13 mmol). The resulted mixture was stirred at 65°C for 16 h. The reaction was quenched with acetic acid aqueous solution (4%, 100 mL) at 0°C, and extracted with ethyl acetate. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford desired mono protected compound **23.3** (1.21 g, 49%).

Benzyl ether 23.5: To a solution of compound **23.3** (0.65 g, 1.33 mmol) and N-phenyltrifluoromethanesulfonimide (0.715 g, 2 mmol) in THF (12 mL) was added cesium carbonate (0.65 g, 2 mmol). The mixture was stirred at room temperature for 3 h. The reaction mixture was filtered through a pad of silica gel and concentrated under reduced pressure. The

residue was purified on silica gel chromatography to give triflate **23.4** (0.85 g). To a solution of 1,3-bis(diphenylphosphino)propane (0.275g, 0.66 mmol) in DMF (10 mL) was added palladium(II) acetate (0.15 g, 0.66 mmol) under argon. This mixture was stirred for 2 min. and then added to triflate **23.4**. After stirring for 2 min., triethylsilane was added and the resulted mixture was stirred for 1.5 h. The solvent was removed under reduced pressure and the residue was purified by chromatography on silica gel to afford compound **23.5** (0.56 g, 89%).

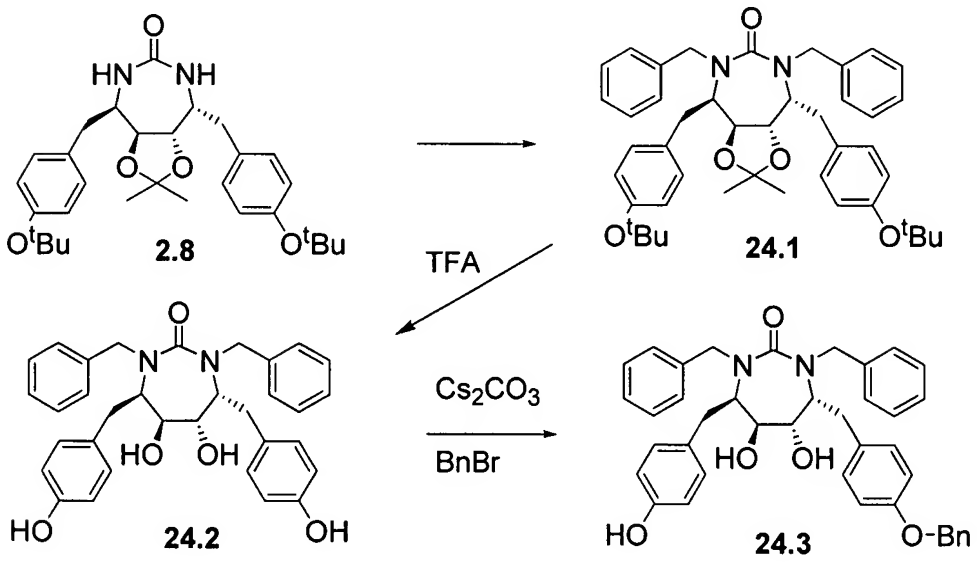
Phenol 23.6: A solution of **23.5** (0.28 g, 0.593 mmol) in ethyl acetate (5 mL) and isopropyl alcohol (5 mL) was treated with 10% Pd/C (0.05g) and stirred under a hydrogen atmosphere (balloon) for 16 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to yield **23.6** (0.22 g, 97%) as a white solid.

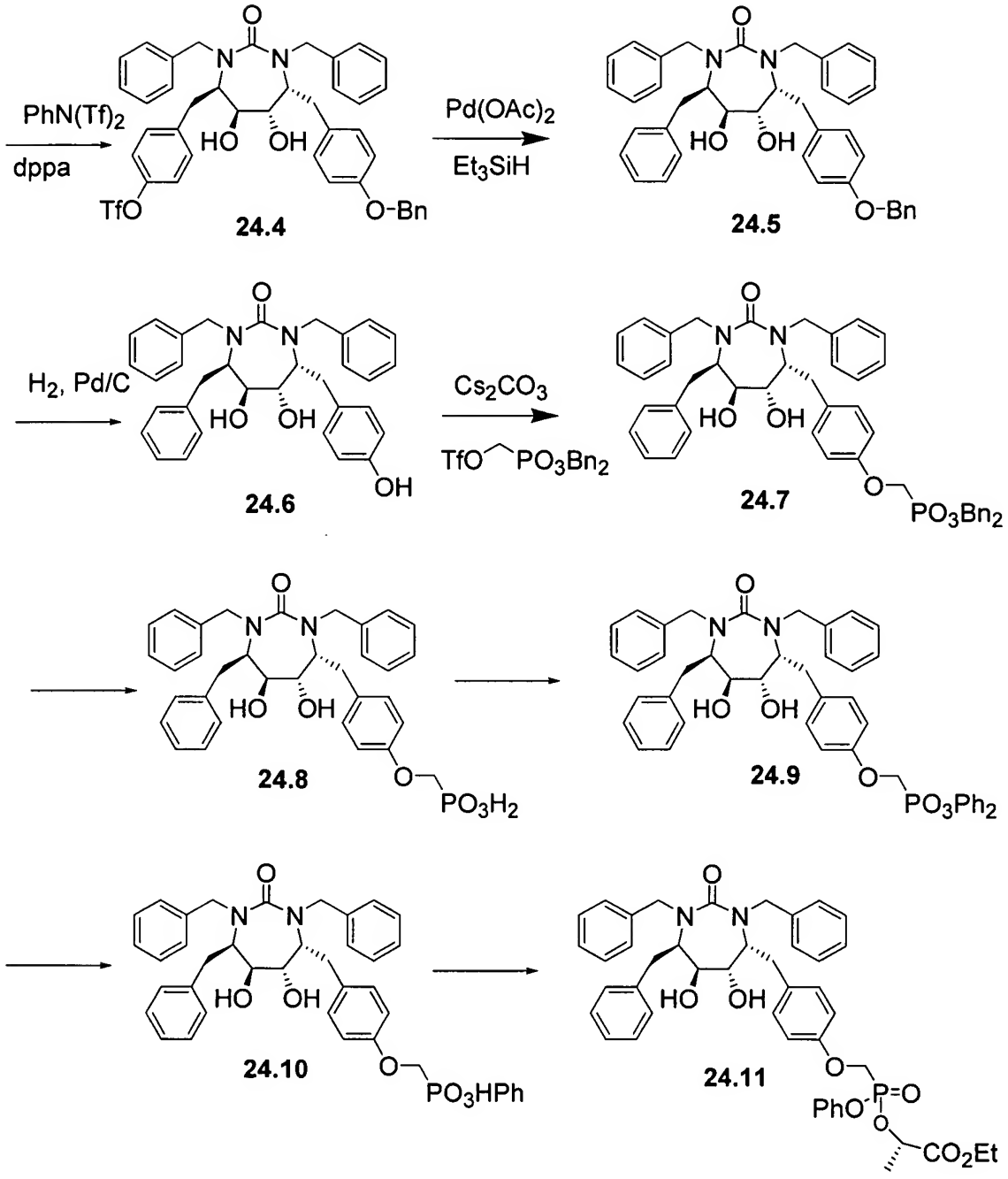
Dibenzyl phosphonate 23.7: To a solution of compound **23.6** (0.215 g, 0.563 mmol) in THF (10 mL) was added dibenzyl triflate **3.11** (0.315 g, 0.74 mmol) and cesium carbonate (0.325g, 1 mmol). The mixture was stirred at room temperature for 2 h, then diluted with ethyl acetate and washed with water. The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford compound **23.7** (0.31 g, 84%).

Diphenyl ester 23.8: A solution of compound **23.7** (0.3 g, 0.457 mmol) and benzyl bromide (0.165 mL, 1.39 mmol) in THF (10 mL) was treated with potassium *tert*-butoxide (1M/THF, 1.2 mL) for 0.5 h. The mixture was diluted with ethyl acetate and washed with HCl (0.2N). The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was dissolved in ethyl acetate and treated with 10% Pd/C (0.05 g) under hydrogen atmosphere (balloon) for 16 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was treated with TFA (1 mL) in methanol (5 mL) for 1 h, and then concentrated under reduced pressure. The residue was dissolved in pyridine (1 mL) and mixed with phenol (0.45 g, 4.8 mmol) and 1,3-dicyclohexylcarbodiimide (0.38 g, 1.85 mmol). The mixture was stirred at 70°C for 2 h, and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate and HCl (0.2N). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was purified by chromatography on silica gel to afford compound **23.8** (0.085 g, 24%).

Mono amidate 23.9: To a solution of **23.8** (0.085g, 0.11 mmol) in acetonitrile (1 mL) was added sodium hydroxide (1N, 0.25 mL) at 0°C. After stirred at 0°C for 1 h, the mixture was acidified with Dowex resin to pH = 3, and filtered. The filtrate was concentrated under reduced pressure. The residue was dissolved in pyridine (0.5 mL) and mixed with L-alanine ethyl ester hydrochloride (0.062 g, 0.4 mmol) and 1,3-dicyclohexyl-carbodiimide (0.125 g, 0.6 mmol). The mixture was stirred at 60°C for 0.5 h, and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate and HCl (0.2N). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was purified by HPLC (C-18, 65% acetonitrile / water) to afford compound **23.9** (0.02 g, 23%). ^{1}H NMR (CDCl3): δ 1.2 (m, 3H), 1.4 (m, 3H), 1.8 (brs, 2H), 2.8-3.1 (m, 6H), 3.5-3.7 (m, 4H), 3.78 (m, 1H), 4.0-4.18 (m, 2H), 4.2-4.4 (m, 3H), 4.9 (m, 2H), 6.8-7.4 (m, 24H). 31P NMR (CDCl3): d 20.9, 19.8. MS: 792 (M+1).

Scheme 24





Di-tert butyl ether 24.1: To a solution of compound **2.8** (0.51 g, 1 mmol) and benzyl bromide (0.43g, 2.5 mmol) in THF (6 mL) was added potassium *tert*-butoxide (1M/THF, 2.5 mL). The mixture was stirred at room temperature for 0.5 h, then diluted with ethyl acetate and washed with water. The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford compound **24.1** (0.62 g, 90%).

Diol 24.2: To a solution of compound **24.1** (0.62 g, 0.9 mmol) in methylene chloride (4 mL) was added TFA (1 mL) and water (0.1 mL). The mixture was stirred for 2 h, and then concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford compound **24.2** (0.443g, 92%).

Benzyl ether 24.3: Compound **24.3** was prepared in 46% yield according to the procedure described in Scheme 23 for the preparation of **23.3**.

Triflate 24.4: Compound **24.4** was prepared in 95% yield according to the procedure described in Scheme 23 for the preparation of **23.4**.

Benzyl ether 24.5: Compound **24.5** was prepared in 93% yield according to the procedure described in Scheme 23 for the preparation of **23.5**.

Phenol 24.6: Compound **24.6** was prepared in 96% yield according to the procedure described in Scheme 23 for the preparation of **23.6** from **23.5**.

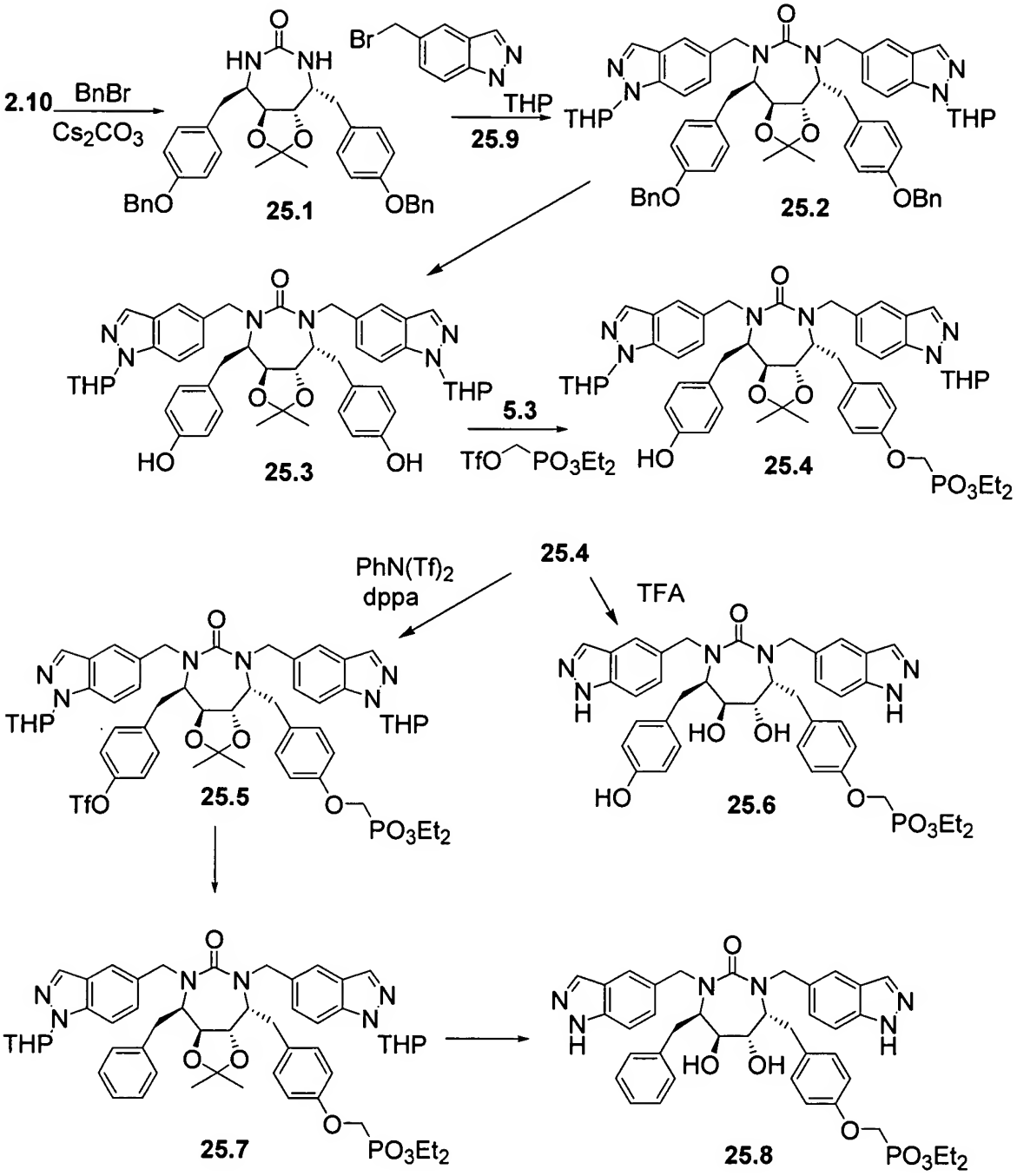
Dibenzyl phosphonate 24.7: Compound **24.7** was prepared in 82% yield according to the procedure described in Scheme 23 for the preparation of **23.7**.

Diacid 24.8: A solution of **24.7** (0.16 g, 0.207 mmol) in ethyl acetate (4 mL) and isopropyl alcohol (4 mL) was treated with 10% Pd/C (0.05g) and stirred under a hydrogen atmosphere (balloon) for 4 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to yield **24.8** (0.125 g, 98%) as a white solid.

Diphenyl ester 24.9: To a solution of compound **24.8** (0.12 g, 0.195 mmol) in pyridine (1 mL) was added phenol (0.19 g, 2 mmol) and 1,3-dicyclohexylcarbodiimide (0.206 g, 1 mmol). The mixture was stirred at 70°C for 2 h, and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate and HCl (0.2N). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was purified by chromatography on silica gel to afford compound **24.9** (0.038 g, 25%).

Mono lactate 24.11: Compound **24.9** was converted, via compound **24.10**, into compound **24.11** in 36% yield according to the procedure described in Scheme 23 for the preparation of **23.9** except utilizing the ethyl lactate ester in place of L-alanine ethyl ester . ^{1}H NMR (CDCl3): δ 1.05 (t, J = 8 Hz, 1.5H), 1.1 (t, J = 8 Hz, 1.5H), 1.45 (d, J = 8 Hz, 1.5H), 1.55 (d, J = 8 Hz, 1.5H), 2.6 (brs, 2H), 2.9-3.1 (m, 6H), 3.5-3.65 (m, 4H), 4.15-4.25 (m, 2H), 4.4-4.62 (m, 2H), 4.9 (m, 2H), 5.2 (m, 1H), 6.9-7.4 (m, 24H). 31P NMR (CDCl3): d 17.6, 15.5. MS: 793 (M+1).

Scheme 25



Dibenzyl ether 25.1: The protection reaction of compound **2.10** with benzyl bromide was carried out in the same manner as described in Scheme 23 to afford compound **25.1**.

Bis indazole 25.2: The alkylation of compound **25.1** with bromide **25.9** was carried out in the same manner as described in Scheme 23 to afford compound **25.2** in 96% yield.

Diol 25.3: A solution of **25.2** (0.18 g, 0.178 mmol) in ethyl acetate (5 mL)) and isopropyl alcohol (5 mL) was treated with 20% Pd(OH)2/C (0.09g) and stirred under a hydrogen atmosphere (balloon) for 24 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to afford **25.3** in quantitative yield.

Diethyl phosphonate 25.4: To a solution of compound **25.3** (0.124 g, 0.15 mmol) in acetonitrile (8 mL) and DMF (1 mL) was added potassium tert-butoxide (0.15 mL, 1M/THF). The mixture was stirred for 10 min. to form a clear solution. Diethyl triflate **5.3** (0.045 g, 0.15 mmol) was added to the reaction mixture. After stirred for 0.5 h, the reaction mixture was diluted with ethyl acetate and washed with HCl (0.1N). The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford compound **25.4** (0.039 g, 55% (based on recovered starting material: 0.064 g, 52%).

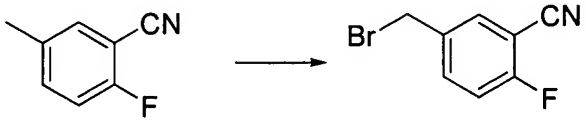
Bisindazole 25.6: A mixture of compound **25.4** (0.027 g), ethanol (1.5 mL), TFA (0.6 mL) and water (0.5 mL) was stirred at 60°C for 18 h. The mixture was concentrated under reduced pressure, and the residue was purified by HPLC to afford compound **25.6** as a TFA salt (0.014 g, 51%). ^{1}H NMR (CD3OD): δ 1.4 (t, J = 8 Hz, 6H), 2.9 (M, 4H), 3.2 (m, 2H), 3.58 (brs, 2H), 3.65 (m, 2H), 4.25 (m, 4H), 4.42 (d, J = 10 Hz, 2H), 4.85 (m, 2H), 6.75 (d, J = 9 Hz, 2H), 6.9 (m, 4H), 7.0 (d, J = 9 Hz, 2H), 7.4-7.6 (m, 6H), 8.1 (brs, 2H). 31P NMR (CD3OD): δ 20.8. MS: 769 (M+1).

Diethyl phosphonate 25.7: Compound **25.4** was converted into compound **25.7** in 76% yield according to the procedures described in Scheme 23 for the conversion of **23.3** into **23.5**.

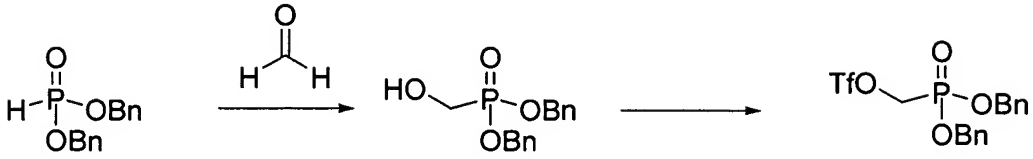
Bis indazole 25.8: Compound **25.7** (0.029 g) was treated in the same manner as compound **25.4** in the preparation of **25.6** to afford compound **25.8** as a TFA salt (0.0175 g, 59%). ^{1}H NMR (CD3OD): δ 1.4 (t, J = 8 Hz, 6H), 3.0 (M, 4H), 3.15 (d, J = 14 Hz, 1H), 3.25 (d, J = 14 Hz, 1H), 3.58 (brs, 2H), 3.65 (m, 2H), 4.25 (m, 4H), 4.42 (d, J = 10 Hz, 2H), 4.85 (m, 2H), 6.9 (d, J = 9 Hz, 2H), 7.0 (d, J = 9 Hz, 2H), 7.1 (d, J = 7 Hz, 2H), 7.2-7.6 (m, 9H), 8.1 (brs, 2H). ^{31}P NMR (CD3OD): δ 20.8. MS: 753 (M+1).

Preparation of Alkylating and Phosphonate Reagents

Scheme 50

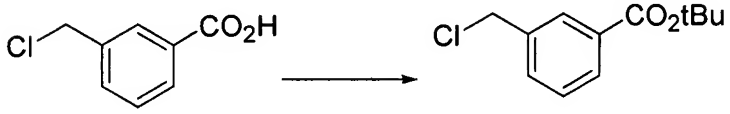


50.1 **3.9**

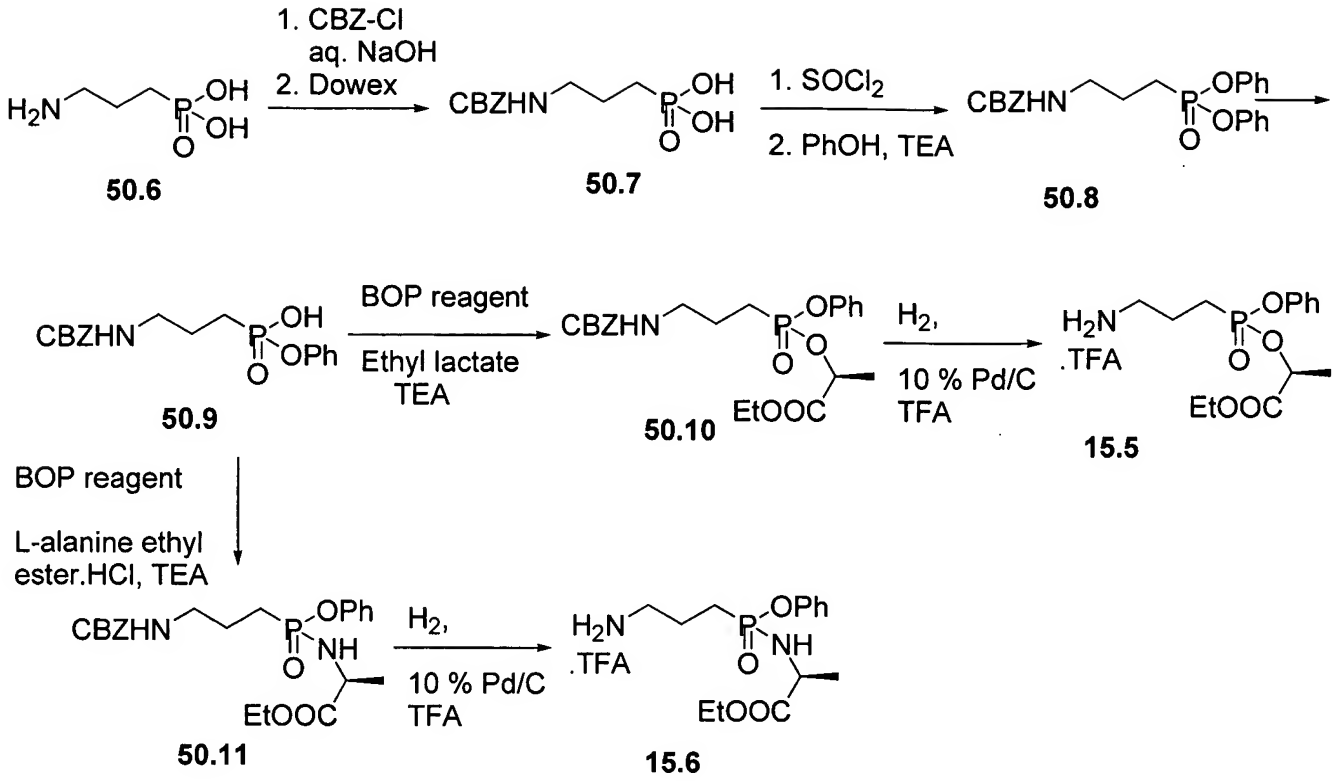


50.2 **50.3** **3.11**

50.4 **6.9**



50.5 **14.5**



3-cyano-4-fluoro-benzylbromide 3.9: The commercially available 2—fluoro-4-methylbenzonitrile **50.1** (10 g, 74 mmol) was dissolved in carbon tetrachloride (50 mL) and then treated with NBS (16 g, 90 mmol) followed by AIBN (0.6 g, 3.7 mmol). The mixture was stirred at 85°C for 30 min and then allowed to cool to room temperature. The mixture was filtered and the filtrate concentrated under reduced pressure. The residue was purified by silica gel eluting with 5-20% ethyl acetate in hexanes to give **3.9** (8.8 g, 56%).

4-benzyloxy benzyl chloride 3.10 is purchased from Aldrich.

Dibenzyl triflate 3.11: To a solution of dibenzyl phosphite **50.2** (100 g, 381 mmol) and formaldehyde (37% in water, 65 mL, 860 mmol) in THF (200 mL) was added TEA (5 mL, 36 mmol). The resulted mixture was stirred for 1 h, and then concentrated under reduced pressure. The residue was dissolved in methylene chloride and hexane (1:1, 300 mL), dried over sodium sulfate, filtered through a pad of silica gel (600 g) and eluted with ethyl acetate and hexane (1:1). The filtrate was concentrated under reduced pressure. The residue **50.3** (95 g) was dissolved in methylene chloride (800 mL), cooled to –78°C and then charged with pyridine (53 mL, 650 mmol). To this cooled solution was slowly added trifluoromethanesulfonic anhydride (120 g, 423 mmol). The resulted reaction mixture was stirred and gradually warmed up to –15°C over

1.5 h period of time. The reaction mixture was cooled down to about – 50°C, diluted with hexane-ethyl acetate (2:1, 500 mL) and quenched with aqueous phosphoric acid (1M, 100 mL) at –10°C to 0°C. The mixture diluted with hexane-ethyl acetate (2:1, 1000 mL). The organic phase was washed with water, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford dibenzyl triflate **3.11** (66 g, 41%) as a colorless oil.

Diethyl triflate 5.3 is prepared as described in *Tetrahedron Lett.* 1986, 27, p1477-1480.

3-Benzyloxybenzylbromide 6.9: To a solution of triphenyl phosphine (15.7 g, 60 mmol) in THF (150 mL) was added a solution of carbon tetrabromide (20 g, 60 mmol) in THF (50 mL). A precipitation was formed and stirred for 10 min. A solution of 3-benzyloxybenzyl alcohol **50.4** (10 g, 46.7 mmol) was added. After stirred for 1.5 h, the reaction mixture was filtered and concentrated under reduced pressure. The majority of triphenyl phosphine oxide was removed by precipitation from ethyl acetate-hexane. The crude product was purified by chromatography on silica gel and precipitation from hexane to give the desired product 3-Benzyloxybenzylbromide **6.9** (10 g, 77%) as a white solid.

t-Butyl-3-chloromethyl benzoate 14.5: A benzene solution (15 ml) of 3-chloromethylbenzoic acid **50.5** (1 g, 5.8 mmol) was heated at reflux, followed by the slow addition of N,N-dimethylforamide-di-t-butylacetal (5 m). The resulting solution was refluxed for 4 h, concentrated under reduced pressure and purified by silica gel column to afford **14.5** (0.8 g, 60 %).

Aminopropyl-diethylphosphonate 14.6 is purchased from Acros.

Aminoethyl-diethylphosphonate oxalate 14.7 is purchased from Acros.

Aminopropyl-phenol-ethyl lactate phosphonate 15.5

N-CBZ-aminopropyl diphenylphosphonate 50.8: An aqueous sodium hydroxide solution (50 mL of 1 N solution, 50 mmol) of 3-aminopropyl phosphonic acid **50.6** (3 g, 1.5 mmol) was reacted with CBZ-Cl (4.1 g, 24 mmol) at room temperature overnight. The reaction mixture was washed with methylene chloride, acidified with Dowex 50wx8-200. The resin was filtered off. The filtrate was concentrated to dryness. The crude N-CBZ-aminopropyl phosphonic acid **50.7** (5.8 mmol) was suspended in CH_3CN (40 mL), and reacted with thionyl chloride (5.2 g, 44 mmol) at reflux for 4 hr, concentrated, and azeotroped with CH_3CN twice. The reaction mixture was redissolved in methylene chloride (20 mL), followed by the addition of

phenol (3.2 g, 23 mmol), was cooled to 0°C. To this 0°C cold solution was added TEA (2.3 g, 23 mmol), and stirred at room temperature overnight. The reaction mixture was concentrated and purified on silica gel column chromatograph to afford **50.8** (1.5 g, 62 %).

Monophenol derivative 50.9: A CH_3CN solution (5 mL) of **50.8** (0.8 g, 1.88 mmol) was cooled to 0°C , and treated with 1N NaOH aqueous solution (4 mL, 4 mmol) for 2 h. The reaction was diluted with water, extracted with ethyl acetate, acidified with Dowex 50wx8-200. The aqueous solution was concentrated to dryness to afford **50.9** (0.56 g, 86%).

Monolactate derivative 50.10: A DMF solution (1 mL) of crude **50.9** (0.17 g, 0.48 mmol), BOP reagent (0.43 g, 0.97 mmol), ethyl lactate (0.12 g, 1 mmol), and DIPEA (0.31 g, 2.4 mmol) was reacted for 4 hr at room temperature. The reaction mixture was partitioned between methylene chloride and 5 % citric acid aqueous solution. The organic solution was separated, concentrated, and purified on preparative TLC to give **50.10** (0.14 g, 66%).

3-Aminopropyl lactate phosphonate 15.5: An ethyl acetate/ethanol solution (10 mL/2 mL) of **50.10** (0.14 g, 0.31 mmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (40 mg) for 3 hr. The catalyst was filtered off. The filtrate was concentrated to dryness to afford **15.5** (0.14 g, quantitative). NMR ($CDCl_3$): δ 8.0-8.2 (b, 3H), 7.1-7.4 (m, 5H), 4.9-5.0 (m, 1H), 4.15-4.3 (m, 2H), 3.1-3.35 (m, 2H), 2.1-2.4 (m, 4H), 1.4 (d, 3H), 1.3 (t, 3H).

Aminopropyl-phenol-ethyl alanine phosphonate 15.6: Compound **15.6** (80 mg) was prepared from the reaction of **50.9** (160 mg, 0.45 mmol) and L-alanine ethyl ester hydrochloride salt (0.11g, 0.68 mmol) in the presence of DIPEA and BOP reagent to give **50.11**, followed by the hydrogenation in the presence of 10% Pd/C and TFA to yield **15.6**. NMR ($CDCl_3$ + ~10 % CD_3OD): δ 8.0-8.2 (b), 7.25-7.35 (t, 2H), 7.1-7.2 (m, 3H), 4.0-4.15 (m, 2H), 3.8-4.0 (m, 1H), 3.0-3.1 (m, 2H), 1.15-1.25 (m, 6H). P NMR ($CDCl_3$ + ~10 % CD_3OD): 32.1 & 32.4 ppm.

Aminopropyl dibenzyl phosphonate 15.7 :

N-BOC-3-aminopropyl phosphonic acid 50.13: A THF-1N aqueous solution (16 mL-16 mL) of 3-aminopropyl phosphonic acid **50.12** (1 g, 7.2 mmol) was reacted with $(BOC)_2O$ (1.7 g, 7.9 mmol) overnight at room temperature. The reaction mixture was concentrated, and partitioned between methylene chloride and water. The aqueous solution was acidified with Dowex 50wx8-200. The resin was filtered off. The filtrate was concentrated to give **50.13** (2.2 g, 92 %).

N-BOC-3-aminopropyl dibenzyl phosphonate 50.14: A CH_3CN solution (10 mL) of **50.13** (0.15 g, 0.63 mmol), cesium carbonate (0.61 g, 1.88 mmol), and benzyl bromide (0.24 g, 1.57 mmol) was heated at reflux overnight. The reaction mixture was cooled to room temperature, and diluted with methylene chloride. The white solid was filtered off, washed thoroughly with methylene chloride. The organic phase was concentrated, and purified on preparative TLC to give **50.14** (0.18 g, 70%). MS: 442 (M + Na).

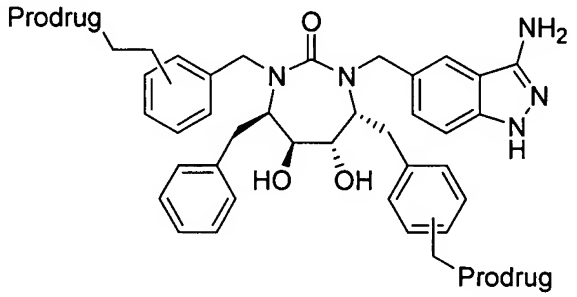
Aminopropyl dibenzyl phosphonate 15.7: A methylene chloride solution (1.6 mL) of **50.14** (0.18 g) was treated with TFA (0.4 mL) for 1 hr. The reaction mixture was concentrated to dryness, and azeotroped with CH_3CN twice to afford **15.7** (0.2 g, as TFA salt). NMR ($CDCl_3$): δ 8.6 (b, 2H), 7.9 (b, 2H), 7.2-7.4 (m, 10H), 4.71-5.0 (2 abq, 4H), 3.0 (b, 2H), 1.8-2 (m, 4H). 31P NMR ($CDCl_3$): 32.0 ppm. F NMR ($CDCl_3$): -76.5 ppm.

Aminomethyl diethylphosphonate 22.8 is purchased from Acros.

Bromomethyl, tetrahydropyran indazole 25.9 is prepared according to *J. Org. Chem.* 1997, 62, p5627.

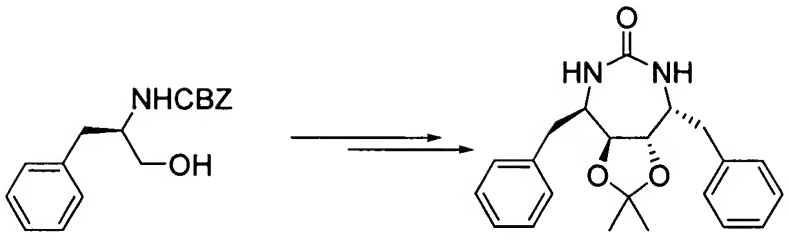
Examples For The Preparation Of Cyclic Carbonyl-Like Phosphonate Protease Inhibitors (CCPPI)

Phosphonamidate Prodrugs



Scheme 1-2	Scaffold Synthesis
Scheme 3-10	P2'-Benzyl ether phosphonates
Scheme 11-13	P2'-Alkyl ether phosphonates
Scheme 14-17	P2'-Benzyl Amide phosphonates
Scheme 18-25	P1-Phosphonates
Scheme 50	Reagents

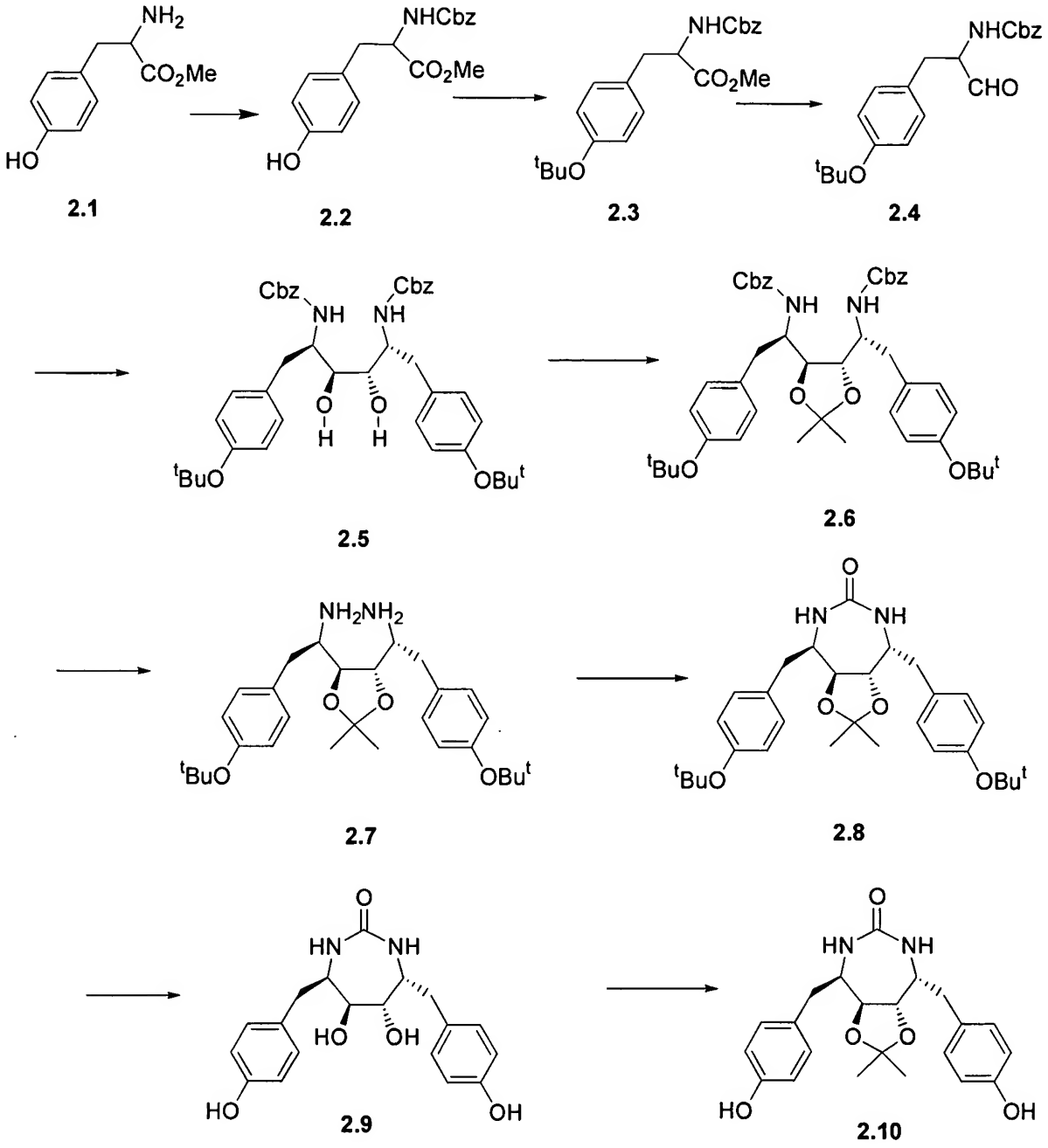
Scheme 1



1 **1.1**

The conversion of **1** to **1.1** is described in *J. Org. Chem.* 1996, 61, p444-450.

Scheme 2



2-Benzyloxycarbonylamino-3-(4-tert-butoxy-phenyl)-propionic acid methyl ester (2.3)

H-D-Tyr-O-me hydrochloride **2.1** (25 g, 107.7 mmol) is dissolved in methylene chloride (150 mL) and aqueous sodium bicarbonate (22 g in 150 mL water), and then cooled to 0°C. To this resulting solution benzyl chloroformate (20 g, 118 mmol) is slowly added. After complete addition, the resulting solution is warmed to room temperature ,and is then stirred for 2 h. The

organic phase is separated, dried over Na_2SO_4, and concentrated under reduced pressure, to give the crude carbamate **2.2** (35g). The crude CBZ-Tyr-OMe product is dissolved in methylene chloride (300 mL) containing concentrated H_2SO_4. Isobutene is bubbled though the solution for 6 h. The reaction is then cooled to 0°C, and neutralized with saturated $NaHCO_3$ aqueous solution. The organic phase is separated, dried, concentrated under reduced pressure, and purified by silica gel column chromatography to afford the tert-butyl ether **2.3** (25.7 g, 62 %).

[2-(4-tert-Butoxy-phenyl)-1-formyl-ethyl]-carbamic acid benzyl ester (2.4)
(Reference J. O. C. 1997, 62, 3884)

To a stirred –78°C methylene chloride solution (60 mL) of **2.3,** DIBAL (82 mL of 1.5 M in toluene, 123 mmol) was added over 15 min. The resultant solution was stirred at –78°C for 30 min. Subsequently, a solution of EtOH/36 % HCl (9/1; 15 mL) is added slowly. The solution is added to a vigorously stirred aqueous HCl solution (600 mL, 1N) at 0°C. The layers are then separated, and the aqueous phase is extracted with cold methylene chloride. The combined organic phases are washed with cold 1N HCl aqueous solution, water, dried over Na_2SO_4, and then concentrated under reduced pressure to give the crude aldehyde **2.4** (20 g, 91 %).

[4-Benzyloxycarbonylamino-1-(4-tert-butoxy-benzyl)-5-(4-tert-butoxy-phenyl)-2,3-dihydroxy-pentyl]-carbamic acid benzyl ester (2.5)

To a slurry of $VCl_3(THF)_3$ in methylene chloride (150 mL) at room temperature is added Zinc powder (2.9 g, 44 mmol), and the resulting solution is then stirred at room temperature for 1 hour. A solution of aldehyde **2.4** (20 g, 56 mmol) in methylene chloride (100 mL) is then added over 10 min. The resulting solution is then stirred at room temperature overnight, poured into an ice-cold H_2SO_4 aqueous solution (8 mL in 200 mL), and stirred at 0°C for 30 min. The methylene chloride solution is separated, washed with 1N HCl until the washing solution is light blue. The organic solution is then concentrated under reduced pressure (solids are formed during concentration), and diluted with hexane. The precipitate is collected and washed thoroughly with a hexane/methylene chloride mixture to give the diol product **2.5**. The filtrate is concentrated under reduced pressure and subjected to silica gel chomatography to afford a further 1.5 g of **2.5**. (Total = 13 g, 65 %).

[1-{5-[1-Benzyloxycarbonylamino-2-(4-tert-butoxy-phenyl)-ethyl]-2,2-dimethyl-[1,3]dioxolan-4-yl}-2-(4-tert-butoxy-phenyl)-ethyl]-carbamic acid benzyl ester (2.6)

Diol **2.5** (5 g, 7 mmol) is dissolved in acetone (120 mL), 2,2-dimethoxypropane (20 mL), and pyridinium p-toluenesulfonate (120 mg, 0.5 mmol). The resulting solution is refluxed for 30 min., and then concentrated under reduced pressure to almost dryness. The resulting mixture is partitioned between methylene chloride and saturated $NaHCO_3$ aqueous solution, dried, concentrated under reduced pressure, and purified by silica gel column chomatography to afford isopropylidene protected diol **2.6** (4.8 g, 92 %).

4,8-Bis-(4-tert-butoxy-benzyl)-2,2-dimethyl-hexahydro-1,3-dioxa-5,7-diaza-azulen-6-one (2.8)

The diol **2.6** is dissolved in EtOAc/EtOH (10 mL/2 mL) in the presence of 10 % Pd/C and hydrogenated at atmospheric pressure to afford the diamino compound **2.7**. To a solution of crude **2.7** in 1,1,2,2-tetrachloroethane is added 1,1-carboxydiimidazole (1.05 g, 6.5 mmol) at room temperature. The mixture is stirred for 10 min, and the resulting solution is then added dropwise to a refluxing 1,1',2,2'-tetrachloroethane solution (150 mL). After 30 min., the reaction mixture is cooled to room temperature, and washed with 5 % citric acid aqueous solution, dried over Na_2SO4, concentrated under reduced pressure, and purified by silica gel column chomatography to afford the cyclourea derivative **2.8** (1.92 g, 60 % over 2 steps).

5,6-Dihydroxy-4,7-bis-(4-hydroxy-benzyl)-[1,3]diazepan-2-one (2.9)

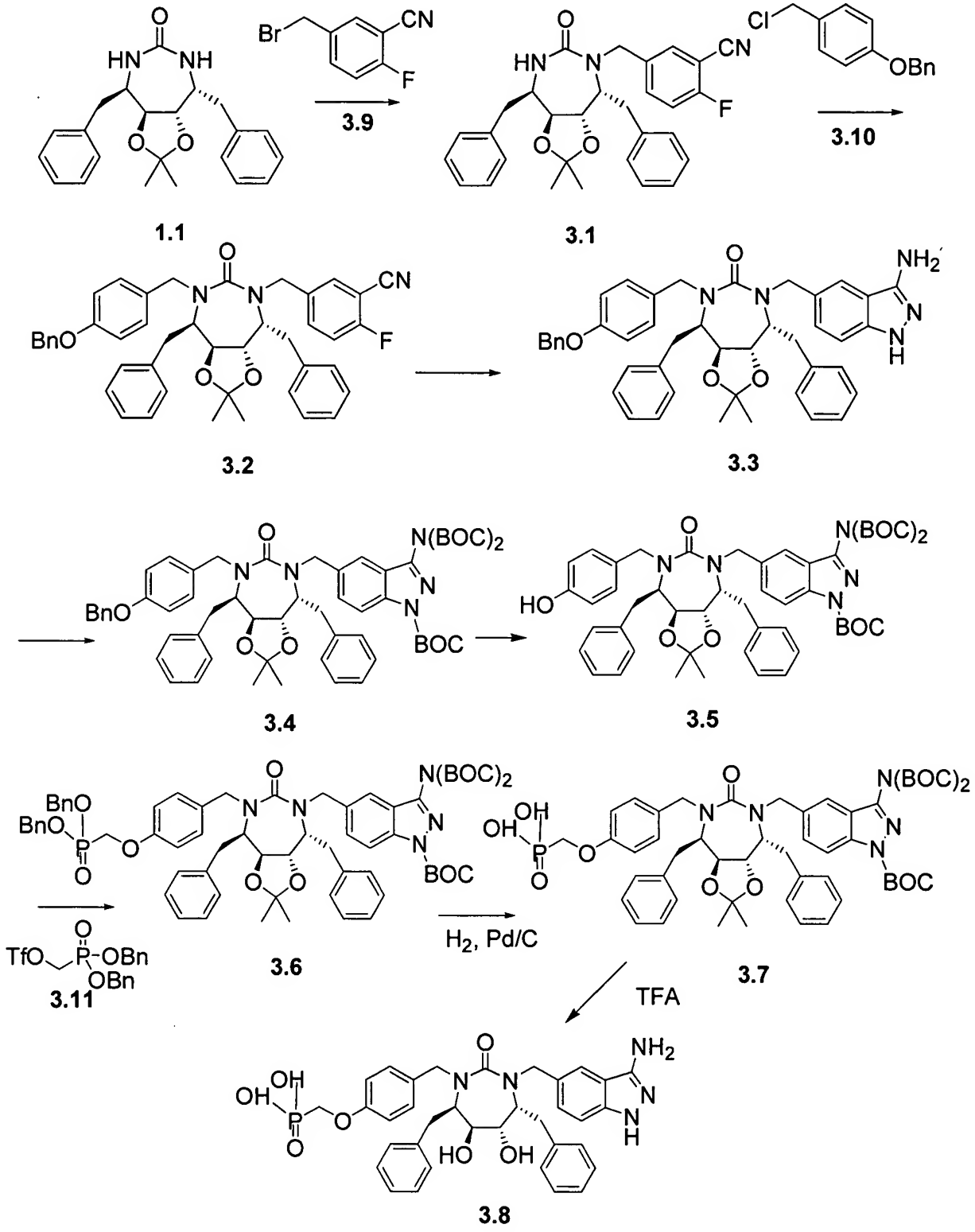
Cyclic Urea **2.8** (0.4 g, 0.78 mmol) was dissolved in dichloromethane (3 mL) and treated with TFA (1 mL). The mixture was stirred at room temperature for 2 h upon which time a white solid precipitated. 2 drops of water and methanol (2 mL) were added and the homogeneous solution was stirred for 1 h and concentrated under reduced pressure. The crude solid, **2.9**, was dried overnight and then used without further purification.

4,8-Bis-(4-hydroxy-benzyl)-2,2-dimethyl-hexahydro-1,3-dioxa-5,7-diaza-azulen-6-one (2.10)

Diol **2.9** (1.8 g, 5.03 mmol) was dissolved in DMF (6 mL) and 2,2-dimethoxypropane (12 mL). P-TsOH (95 mg) was added and the mixture stirred at 65°C for 3 h. A vacuum was applied to remove water and then the mixture was stirred at 65°C for a further 1 h. The excess

dimethoxypropane was then distilled and the remaining DMF solution was then allowed to cool. The solution of acetonide **2.10** can then used without further purification in future reactions.

Scheme 3



3-Cyano-4-fluorobenzyl urea 3.1: A solution of urea **1.1** (1.6 g, 4.3 mmol) in THF was treated with sodium hydride (0.5 g of 60 % oil dispersion, 13 mmol). The mixture was stirred at room temperature for 30 min and then treated with 3-cyano-4-fluorobenzyl bromide **3.9** (1.0 g, 4.8 mmol). The resultant solution was stirred at room temperature for 3 h, concentrated under reduced pressure, and then partitioned between CH_2Cl_2 and saturated brine solution containing 1 % citric acid. The organic phase was separated , dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 15-25% ethyl acetate in hexanes to yield urea **3.1** (1.5 g, 69 %) as a white form.

Benzyl ether 3.2: A solution of **3.1** (0.56 g, 1.1 mmol) in DMF (5 mL) was treated with sodium hydride (90 mg of 60 % oil dispersion, 2.2 mmol) and the resultant mixture stirred at room temperature for 30 min. 4-Benzyloxy benzyl chloride **3.10** (0.31 g, 1.3 mmol) was added and the resultant solution stirred at room temperature for 3 h. The mixture was concentrated under reduced pressure and then partitioned between CH_2Cl_2 and saturated brine solution. The organic phase was separated, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by silica gel eluting with 1-10% ethyl acetate in hexanes to yield compound **3.2** (0.52 g, 67 %) as white form.

Indazole 3.3: Benzyl ether **3.2** (0.51 g, 0.73 mmol) was dissolved in n-butanol (10 mL) and treated with hydrazine hydrate (1 g, 20 mmol). The mixture was refluxed for 4 h and then allowed to cool to room temperature. The mixture was concentrated under reduced pressure and the residue was then partitioned between CH_2Cl_2 and 10 % citric acid solution. The organic phase was separated, concentrated under reduced pressure, and then purified by silica gel column eluting with 5% methanol in CH_2Cl_2 to afford indazole **3.3** (0.42 g, 82 %) as white solid.

Boc-indazole 3.4: A solution of indazole **3.3** (0.4 g, 0.59 mmol) in CH_2Cl_2 (10 mL) was treated with diisopropylethylamine (0.19 g, 1.5 mmol), DMAP (0.18 g, 1.4 mmol), and di-tert-butyl dicarbonate (0.4 g, 2 mmol). The mixture was stirred at room temperature for 3 h and then partitioned between CH_2Cl_2 and 5 % citric acid solution. The organic phase was separated, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 2% methanol in CH_2Cl_2 to afford **3.4** (0.42 g, 71 %).

Phenol 3.5: A solution of **3.4** (300 mg, 0.3 mmol) in ethyl acetate (10 mL) and methanol (10 mL) was treated with 10 % Pd/C (40 mg) and stirred under a hydrogen atmosphere (balloon)

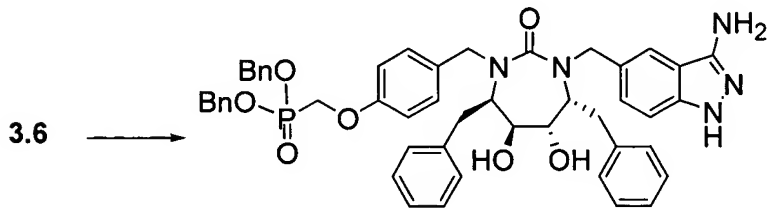
for 16 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to yield **3.5** as a white powder. This was used without further purification.

Dibenzyl ester 3.6: A solution of **3.5** (0.1 mmol) in THF (5 mL) was treated with dibenzyl triflate **3.11** (90 mg, 0.2 mmol), and cesium carbonate (0.19 g, 0.3 mmol). The mixture was stirred at room temperature for 4 h and then concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and saturated brine. The organic phase was separated, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 20-40% ethyl acetate in hexanes to afford **3.6** (70 mg, 59 %). ^{1}H NMR ($CDCl_3$): δ 8,07 (d, 1H), 7.20-7.43 (m, 16H), 7.02-7.15 (m, 8 H), 6.80 (d, 2H), 5.07-5.18 (m, 4H), 5.03 (d, 1H), 4.90 (d, 1H), 4.20 (d, 2H), 3.74-3.78 (m, 4H), 3.20 (d, 1H), 3.05 (d, 1H) 2.80-2.97 (m, 4H), 1.79 (s, 9H), 1.40 (s, 18H), 1.26 (s, 6H); ^{31}P NMR ($CDCl_3$): 20.5 ppm.

Phosphonic acid 3.7: A solution of dibenzylphosphonate **3.6** (30 mg) in EtOAc (10 mL) was treated with 10% Pd/C (10 mg) and the mixture was stirred under a hydrogen atmosphere (balloon) for 3 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to afford phosphonic acid **3.7**. This was used without further purification.

Phosphonic acid 3.8: The crude phosphonic acid **3.7** was dissolved in CH_2Cl_2 (2 mL) and treated with trifluoroacetic acid (0.4 mL). The resultant mixture was stirred at room temperature for 4 h. The mixture was concentrated under reduced pressure and then purified by preparative HPLC (35 % CH_3CN/65 % H_2O) to afford the phosphonic acid **3.8** (9.4 mg, 55 %). ^{1}H NMR (CD_3OD): δ 7.71 (s, 1H), 7.60 (d, 1H), 6.95-7.40 (m, 15H), 4.65 (d, 2H), 4.17 (d, 2H), 3.50-3.70 (m, 3H), 3.42 (d, 1H), 2.03-3.14 (m, 6H); ^{31}P NMR ($CDCl_3$): 17.30.

Scheme 4

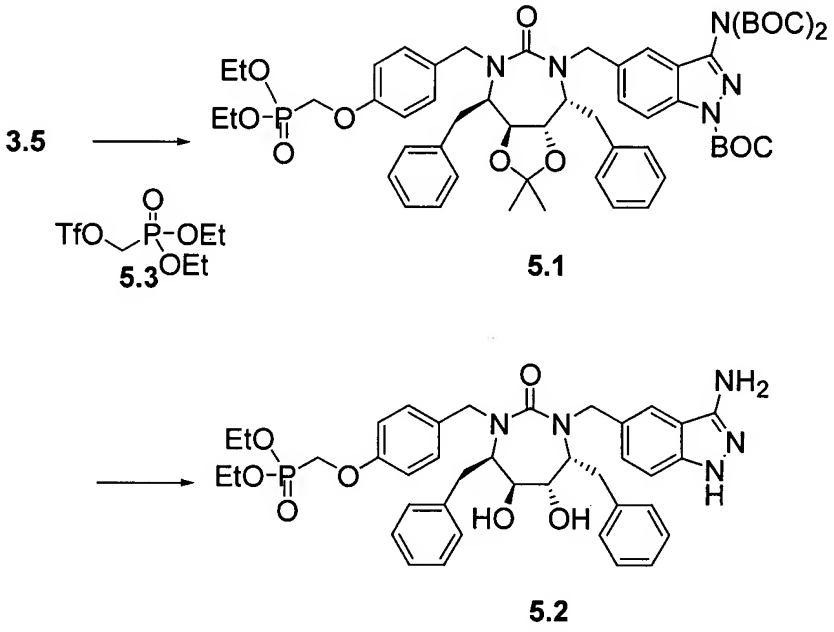


4.1

Dibenzylphosphonate 4.1: A solution of **3.6** (30 mg, 25 μmol) in CH_2Cl_2 (2 mL) was treated with TFA (0.4 mL) and the resultant mixture was stirred at room temperature for 4 h.

The mixture was concentrated under reduced pressure and the residue was purified by silica gel eluting with 50% ethyl acetate in hexanes to afford **4.1** (5 mg, 24%). ^{1}H NMR ($CDCl_3$): δ 6.96-7.32 (m, 25H), 6.95 (d, 2H), 5.07-5.18 (m, 4H), 4.86 (d, 1H), 4.7 5 (d, 1H), 4.18 (d, 2H), 3.40-3.62 (m, 4H), 3.25 (d, 1H), 2.80-3.15 (m, 6H); ^{31}P NMR ($CDCl_3$) 20.5 ppm; MS : 852 (M + H), 874 (M + Na).

Scheme 5

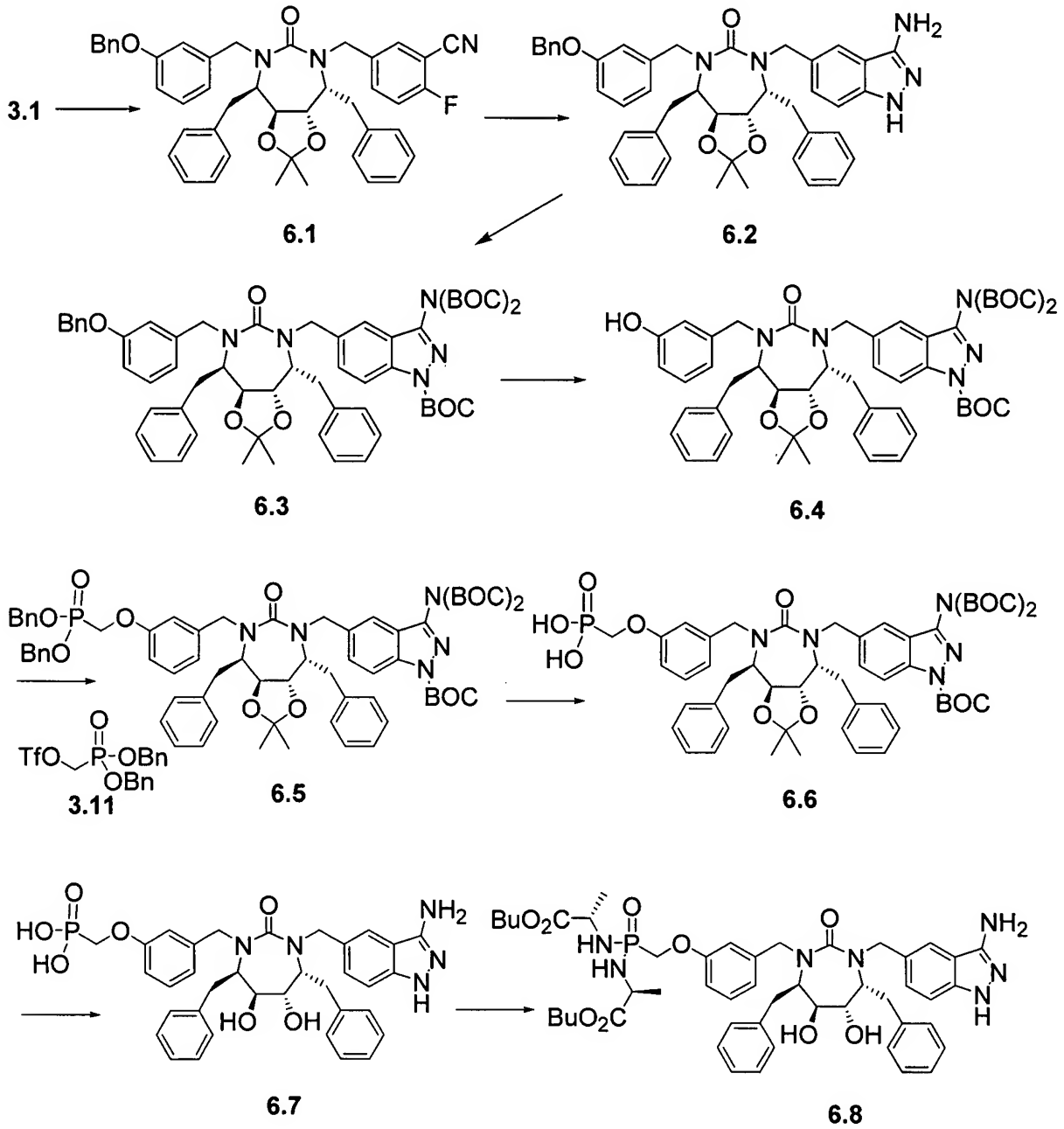


Diethylphosphonate 5.1: A solution of phenol **3.5** (48 mg, 52 μmol) in THF (5 mL) was treated with triflate **5.3** (50 mg, 165 μmol), and cesium carbonate (22 mg, 0.2 mmol). The resultant mixture was stirred at room temperature for 5 h and then concentrated under reduced pressure. The residue was partitioned between CH_2Cl_2 and saturated brine. The organic phase was separated, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 7% methanol in CH_2Cl_2 to afford **5.1** (28 mg, 50 %). ^{1}H NMR ($CDCl_3$): δ 8,06 (d, 1H), 7.30-7.43 (m, 7H), 7.02-7.30 (m, 7 H), 6.88 (d, 2H), 5.03 (d, 1H), 4.90 (d, 1H), 4.10-4.25 (m, 6H), 3.64-3.80 (m, 4H), 3.20 (d, 1H), 3.05 (d, 1H) 2.80-2.97 (m, 4H), 1.79 (s, 9H), 1.20-1.50 (m, 30H); ^{31}P NMR ($CDCl_3$): 18.5 ppm; MS :1068 (M + H), 1090 (M + Na).

Diethylphosphonate 5.2: A solution of **5.1** (28 mg, 26 μmol) in CH_2Cl_2 (2 mL) was treated with TFA (0.4 mL) and the resultant mixture was stirred at room temperature for 4 hrs.

The mixture was concentrated under reduced pressure and the residue was purified by silica gel to afford **5.2** (11 mg, 55 %). ^{1}H NMR ($CDCl_3$ + 10 % CD_3OD): δ 6.96-7.35 (m, 15H), 6.82 (d, 2H), 4.86(d, 1H), 4.75 (d, 1H), 4.10-4.23 (M, 6H), 3.40-3.62 (m, 4H), 2.80-3.20 (m), 1.31 (t, 6 H); ^{31}P NMR ($CDCl_3$ + 10 % CD_3OD): 19.80 ppm; MS : 728 (M + H).

Scheme 6



3-Benzyloxybenzyl urea 6.1: The urea **3.1** (0.87 g, 1.7 mmol) was dissolved in DMF and treated with sodium hydride (60% dispersion, 239 mg, 6.0 mmol) followed by m-benzyloxybenzylbromide **6.9** (0.60 g, 2.15 mmol). The mixture was stirred for 5 h and then diluted with ethyl acetate. The solution was washed with water, brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 25% ethyl acetate in hexanes to afford urea **6.1** (0.9 g, 75%).

Indazole 6.2: The urea **6.1** (41 mg, 59 μmol) was dissolved in n-butanol (1.5 mL) and treated with hydrazine hydrate (100 μL, 100 mmol). The mixture was refluxed for 2 h and then allowed to cool. The mixture was diluted with ethyl acetate, washed with 10% citric acid solution, brine, saturated $NaHCO_3$, and finally brine again. The organic phase was dried over sodium sulfate, filtered and concentrated under reduced pressure to give the crude product **6.2** (35 mg, 83%). (*Chem. Biol.* 1998, 5, 597-608).

Boc-indazole 6.3: The indazole **6.2** (1.04 g, 1.47 mmol) was dissolved in CH_2Cl_2 (20 mL) and treated with di-t-butyl dicarbonate (1.28 g, 5.9 mmol), DMAP (0.18 g, 1.9 mmol) and DIPEA (1.02 ml, 9.9 mmol). The mixture was stirred for 3 h and then diluted with ethyl acetate. The solution was washed with 5% citric acid solution, $NaHCO_3$, brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with 50% ethyl acetate in hexanes to give **6.3** (0.71 g, 49%).

Phenol 6.4: Compound **6.3** (20 mg, 0.021 mmol) was dissolved in MeOH (1 mL) and EtOAc (1 mL) and treated with 10% Pd/ C catalyst (5 mg). The mixture was stirred under a hydrogen atmosphere (balloon) until completion. The catalyst was removed by filtration and the filtrate concentrated under reduced pressure to afford compound **6.4** (19 mg, 100%).

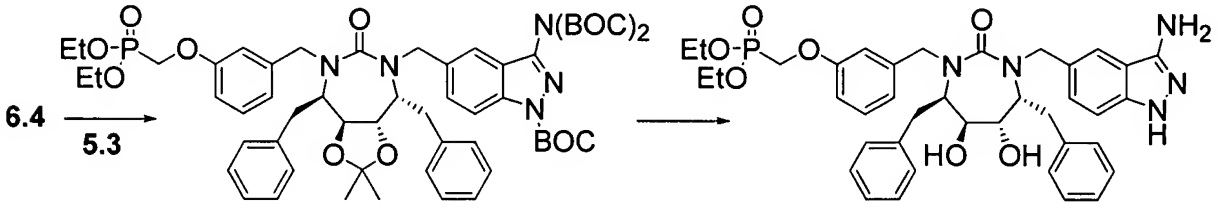
Dibenzyl phosphonate 6.5: A solution of compound **6.4** (0.34 g, 0.37 mmol) in acetonitrile (5 mL) was treated with Cs_2CO_3 (0.36 g, 1.1 mmol) and triflate **3.11** (0.18 mL, 0.52 mmol). The reaction mixture was stirred for 1 h. The reaction mixture was filtered and the filtrate was then concentrated under reduced pressure. The residue was re-dissolved in EtOAc, washed with water, saturated $NaHCO_3$, and finally brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by silica gel eluting with hexane: EtOAc (1:1) to afford compound **6.5** (0.32 g, 73%).

Phosphonic acid 6.6: Compound **6.5** (208 mg, 0.174 mmol) was treated in the same manner as benzyl phosphonate **3.6** in the preparation of phosphonate diacid **3.7**, except MeOH was used as the solvent, to afford compound **6.6** (166 mg, 94%).

Phosphonic acid 6.7: Compound **6.6** (89 mg, 0.088 mmol) was treated according to the conditions described in Scheme 3 for the conversion of **3.7** into **3.8**. The residue was purified by preparative HPLC eluting with a gradient of 90% methanol in 100 mM TEA bicarbonate buffer and 100% TEA bicarbonate buffer to afford phosphonic acid **6.7** (16 mg, 27%)

Bisamidate 6.8: Triphenylphosphine (112 mg, 0.43 mmol) and aldrithiol-2 (95 mg, 0.43 mmol) were mixed in dry pyridine (0.5 mL). In an adjacent flask the diacid **6.7** (48 mg, 0.71 mmol) was suspended in dry pyridine (0.5 mL) and treated with DIPEA (0.075 mL 0.43 mmol) and L-AlaButyl ester hydrochloride (78 mg, 0.43 mmol) and finally the triphenylphosphine, aldrithiol-2 mixture. The reaction mixture was stirred under nitrogen for 24 h then concentrated under reduced pressure. The residue was purified by preparative HPLC eluting with a gradient of 5% to 95% acetonitrile in water. The product obtained was then further purified by silica gel eluting with CH_2Cl_2 : MeOH (9:1) to give compound **6.8** (9 mg, 14%).

Scheme 7

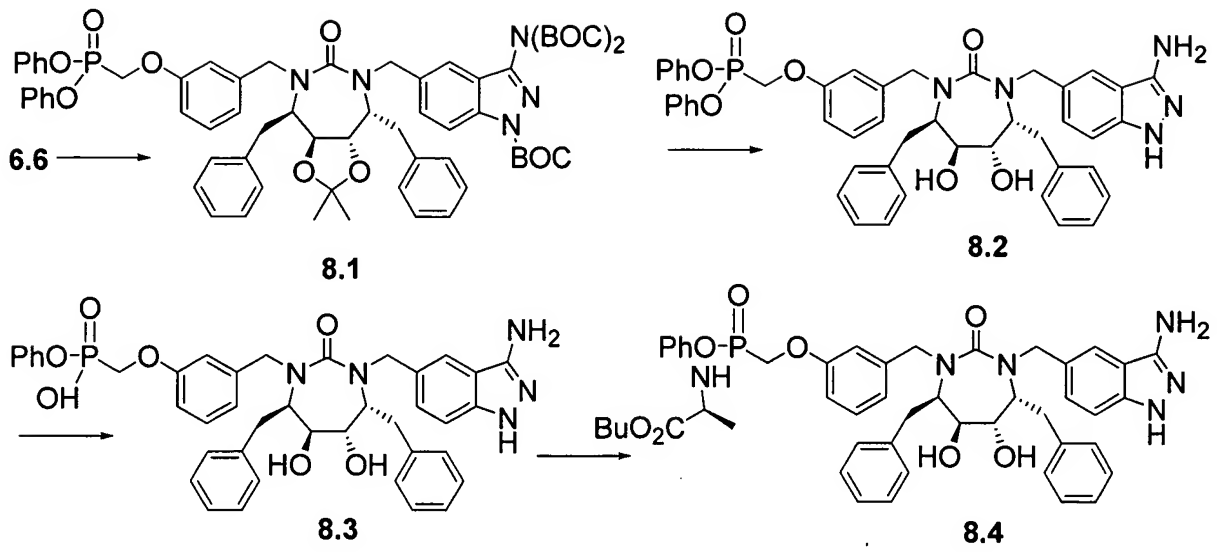


7.1 **7.2**

Diethyl phosphonate 7.1: Compound **6.4** (164 mg, 0.179 mmol) was treated according to the procedure used to generate compound **6.5** except triflate **5.3** was used in place of triflate **3.11** to afford compound **7.1** (142 mg, 74%).

Diethylphosphonate 7.2: Compound **7.1** (57 mg, 0.053 mmol) was treated according to the conditions used to form **6.7** from **6.6**. The residue formed was purified by silica gel eluting with CH_2Cl_2 : MeOH (9:1) to afford compound **7.2** (13 mg, 33%).

Scheme 8



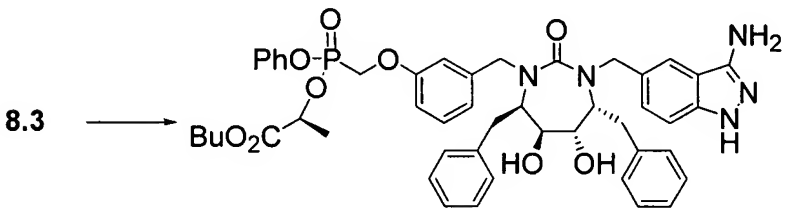
Diphenylphosphonate 8.1: A solution of **6.6** (0.67g, 0.66 mmol) in pyridine (10 mL) was treated with phenol (0.62 g, 6.6 mmol) and DCC (0.82 mg, 3.9 mmol). The resultant mixture was stirred at room temperature for 5 min and then the solution was heated at 70°C for 3 h. The mixture was allowed to cool to room temperature and then diluted with EtOAc and water (2 mL). The resultant mixture was stirred at room temperature for 30 min and then concentrated under reduced pressure. The residue was triturated with CH_2Cl_2, and the white solid that formed was removed by filtration. The filtrate was concentrated under reduced pressure and the resultant residue was purified by silica gel eluting with 30% ethyl acetate in hexanes to yield **8.1** (0.5 g, 65 %). ^{1}H NMR ($CDCl_3$): δ 8,08 (d, 1H), 7.41 (d, 1H), 7.05-7.35 (m, 22H), 6.85 (d, 2H), 6.70 (s, 1H). 5.19 (d, 1H), 5.10 (d, 1H), 4.70 (d, 2H), 3.70-3.90 (m, 4H), 3.20 (d, 1H), 3.11 (d, 1H), 2.80-2.97 (m, 4H), 1.79 (s, 9H), 1.40 (s, 18H), 1.30 (s, 6H); ^{31}P NMR ($CDCl_3$): 12.43 ppm.

Diphenylphosphonate 8.2: A solution of **8.1** (0.5 g, 0.42 mmol) in CH_2Cl_2 (4 mL) was treated with TFA (1 mL) and the resultant mixture was stirred at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and azeotroped twice with CH_3CN. The residue was purified by silica gel eluting with 5% methanol in CH_2Cl_2 to afford diphenylphosphonate **8.2** (0.25 g, 71 %). ^{1}H NMR ($CDCl_3$): δ 7.03-7.40 (m, 21H), 6.81-6.90 (m, 3H), 4.96 (d, 1H), 4.90 (d, 1H) 4.60-4.70 (m, 2H), 3.43-3.57 (m, 4H), 3.20 (d, 1H), 2.80-2.97 (m, 5H); ^{31}P NMR ($CDCl_3$): 12.13 ppm; MS : 824 (M + H).

Monophenol 8.3: The monophenol **8.3** (124 mg, 68 %) was prepared from the diphenol **8.2** by treating with 1N NaOH in acetonitrile at 0°C.

Monoamidate 8.4: To a pyridine solution (0.5 mL) of **8.3** (40 mg, 53 μmol), n-butyl amidate HCl salt (116 mg, 640 μmol), and DIPEA (83 mg, 640 μmol) was added a pyridine solution (0.5 mL) of triphenyl phosphine (140 mg, 640 μmol), and aldrithiol-2 (120 mg, 640 μmol). The resulting solution was stirred at 65°C overnight, worked up, and purified by preparative TLC twice to give **8.4** (1.8 mg). δ 4.96 (d, 1H), 4.90 (d, 1H) 4.30-4.6 (m, 2H), 3.9-4.2 (m, 2H), 3.6-3.70 (m, 4H), 3.2-3.3 (d, 1H), 2.80-3.1 (m, 4H); MS: 875 (M + H) & 897 (M + Na).

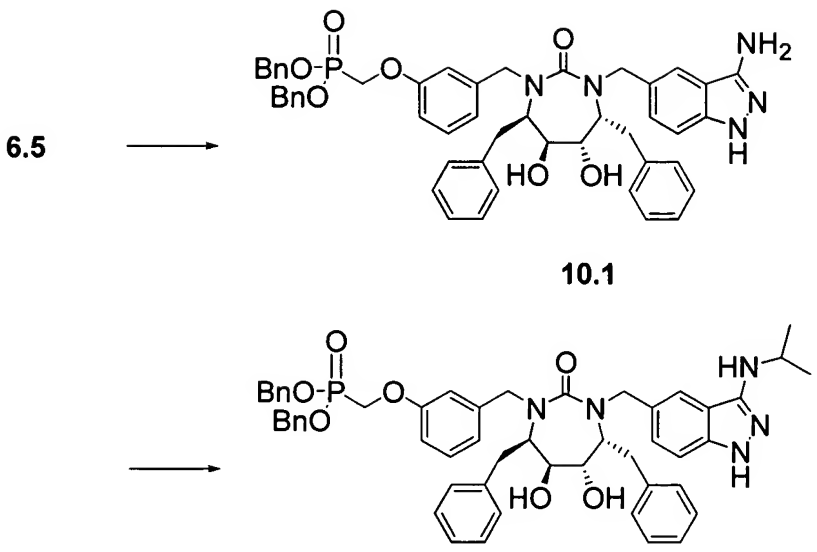
Scheme 9



9.1

Monolactate **9.1**: The monolactate **9.1** is prepared from **8.3** using the conditions described above for the preparation of the monoamidate **8.4** except n-butyl lactate was used in place of n-butyl amidate HCl salt.

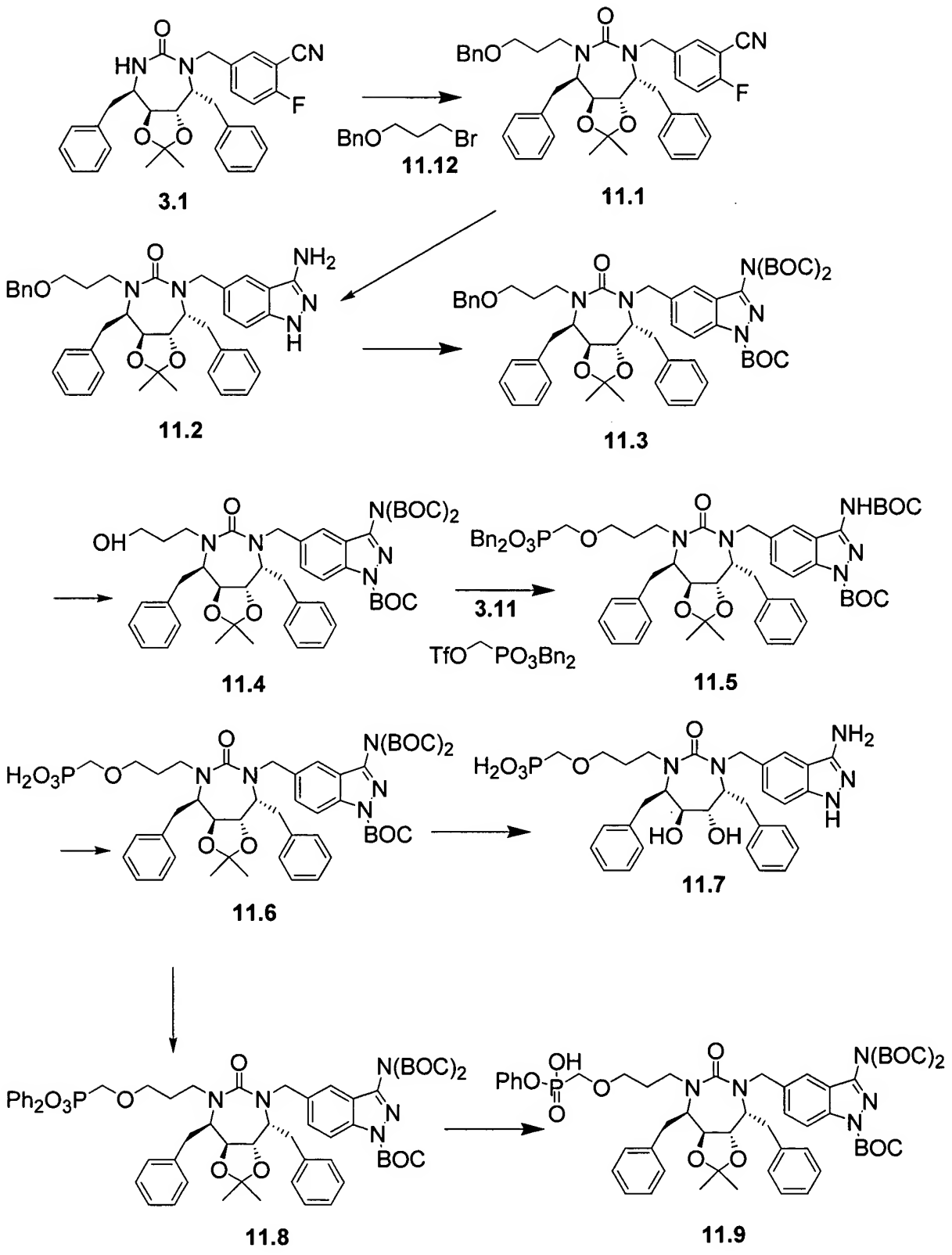
Scheme 10

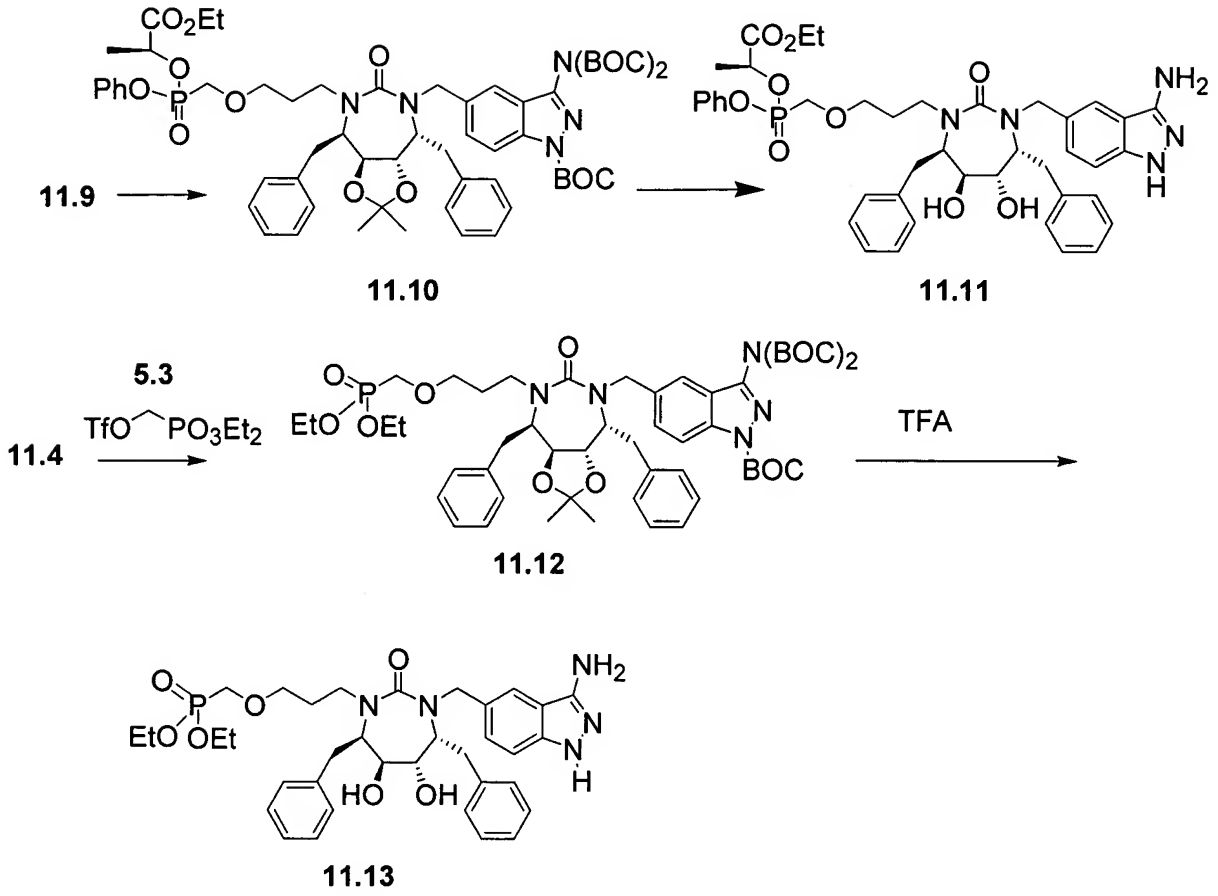


Dibenzylphosphonate 10.1: Compound **6.5** (16 mg, 0.014 mmol) was dissolved in CH_2Cl_2 (2 mL) and cooled to 0°C. TFA (1 mL) was added and the reaction mixture was stirred for 0.5 h. The mixture was then allowed to warm to room temperature for 2 h. The reaction mixture was concentrated under reduced pressure and azeotroped with toluene. The residue was purified by silica gel eluting with CH_2Cl_2 : MeOH (9:1) to afford compound **10.1** (4 mg, 32%).

Isopropylamino indazole 10.2: Compound **10.1** (30 mg, 0.35 mmol) was treated with acetone according to the method of Henke *et al.* (*J. Med. Chem.* 40 17 (1997) 2706-2725) to yield **10.2** as a crude residue. The residue was purified by silica gel eluting with CH_2Cl_2 : MeOH (93:7) to afford compound **10.2** (3.4 mg, 10%).

Scheme 11





Benzyl ether 11.1: A DMF solution (5 mL) of **3.1** (0.98 g, 1.96 mmol) was treated with NaH (0.24 g of 60 % oil dispersion, 6 mmol) for 30 min, followed by the addition of sodium iodide (0.3 g, 2 mmol), and benzoxypropyl bromide (0.55 g, 2.4 mmol). After the reaction for 3 h at room temperature, the reaction mixture was partitioned between methylene chloride and saturated NaCl, dried, and purified to give **11.1** (0.62 g, 49 %).

Aminoindazole 11.2: A n-butanol solution (10 mL) of **11.1** (0.6 g, 0.92 mmol) and hydrazine hydrate (0.93 g, 15.5 mmol) was heated at reflux for 4 h. The reaction mixture was concentrated under reduced pressure to give crude **11.2** (~0.6 g).

Tri-BOC-Aminoindazole 11.3: A methylene chloride solution (10 mL) of crude **11.2**, DIPEA (0.36 g, 2.8 mmol), $(BOC)_2O$ (0.73 g, 3.3 mmol), and DMAP (0.34 g, 2.8 mmol) was stirred for 5 h at room temperature, partitioned between methylene chloride and 5 % citric acid solution, dried, purified by silica gel column chomatography to give **11.3** (0.51 g, 58 %, 2 steps).

3-Hydroxypropyl cyclic urea 11.4: An ethyl acetate/ethanol solution (30 mL/5 mL) of **11.3** (0.5 g, 0.52 mmol) was hydrogenated at 1 atm in the presence of 10 % Pd/C (0.2 g) for 4 h.

The catalyst was removed by filtration. The filtrate was then concentrated under reduced pressure to afford crude **11.4** (0.44 g, 98 %).

Dibenzyl phosphonate 11.5: A THF solution (3 mL) of **11.4** (0.5 g, 0.57 mmol) and triflate dibenzyl phosphonate **3.11** (0.37 g, 0.86 mmol) was cooled to –3°C, followed by addition of n-BuLi (0.7 mL of 2.5 M hexane solution, 1.7 mmol). After 2 h reaction, the reaction mixture was partitioned between methylene chloride and saturated NaCl solution, concentrated under reduced pressure. The residue was redissolved in methylene chloride (10 mL), and reacted with $(BOC)_2O$ (0.15 g, 0.7 mmol) in the presence of DMAP (0.18 g, 0.57 mmol), DIPEA (0.18 g, 1.38 mmol) for 2 h at room temperature. The reaction mixture was worked up, and purified by silica gel chromatography to give **11.5** (0.25 g, 43 %).

Phosphonic diacid 11.7: An ethyl acetate solution (2 mL) of **11.5A** (11 mg, 10.5 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (10 mg) for 6 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give crude **11.6**. The crude **11.6** was redissolved in methylene chloride (1 mL) and treated with TFA (0.2 mL) for 4 h at room temperature. The reaction mixture was concentrated under reduced pressure and purified by HPLC to give **11.7** (2 mg, 30%).

NMR (CD_3OD): δ 7.1-7.3 (m, 11H), 7.0-7.1 (d, 2H), 4.95 (d, 1H), 3.95-4.1 (d, 1H), 2.9 - 3.3 (m, 4H), 2.3-2.45 (m, 1H), 1.6-1.8 (m, 2H). P NMR (CD_3OD):15.5 ppm. MS: 624 (M + 1).

Diphenyl phosphonate 11.8: A pyridine solution (1 mL) of **11.6** (0.23 g, 0.23 mmol), phenol (0.27 g, 2.8 mmol), and DCC (0.3 g, 1.4 mmol) was stirred for 5 min. at room temperature, then reacted at 70°C for 3 h. The reaction mixture was cooled to room temperature, concentrated under reduced pressure, and purified by silica gel column chromatograph to afford **11.8** (0.11g, 41 %).

Monophenyl phosphonate 11.9: An acetonitrile solution (2 mL) of **11.8** (0.12 g, 0.107 mmol) at 0°C was treated with 1N sodium hydroxide aqueous solution (0.2 mL) for 1.5 h., then acidified with Dowex (50wx8-200, 120 mg). The Dowex was removed by filtration, and the filtrate was concentrated under reduced pressure. The residue was triturated with 10 % EtOAc/90 % hexane twice to afford **11.9** (90 mg, 76 %) as a white solid.

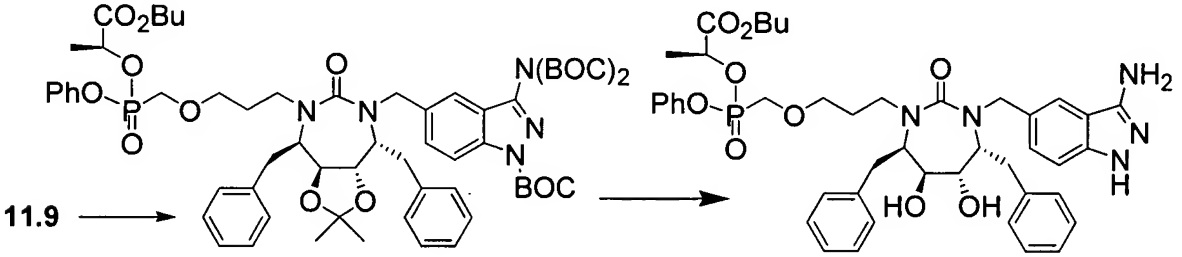
Mono-ethyl lactate phosphonate 11.10: A pyridine solution (0.3 mL) of **11.9** (33 mg, 30 μmol), ethyl lactate (41 mg, 340 μmol), and DCC (31 mg, 146 μmol) was stirred at room temperature for 5 min, then reacted at 70°C for 1.5 h. The reaction mixture was concentrated

under reduced pressure, partitioned between methylene chloride and saturated NaCl solution, and purified by silica gel chromatography to give **11.10** (18 mg, 50 %).

Ethyl lactate phosphonate 11.11: A methylene chloride solution (0.8 mL) of **11.10** (18 mg, 15.8 μmol) was treated with TFA (0.2 mL) for 4 h, and then concentrated under reduced pressure. The residue was purified by preparative TLC to give **11.11** (6 mg, 50 %). NMR ($CDCl_3$ + ~10 %CD_3OD): δ 7.0-7.3 (m, 16 H), 6.8-7.0 (m, 2H), 4.9-5.0 (m, 1H), 4.75 (d, 1H), 4.1-4.2 (m, 2H). 3.5-4.0 (m, 10H), 2.18-2.3. (m, 1H), 1.6-1.7 (m, 1), 1.47 & 1.41 (2d, 3H), 1.22 (t, 3H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 19.72 & 17.86 ppm.

Diethyl phosphonate 11.13: Compound **11.13** (6 mg) was prepared as described above in Scheme 5 from **11.4** (30 mg, 34 μmol) and triflate phosphonate **5.3** (52 mg, 172 μmol), followed by TFA treatment. NMR ($CDCl_3$ + ~10 %CD_3OD): δ 7.1-7.32 (m, 11 H), 6.9-7.0 (d, 2H), 4.75 (d, 1H), 4.1-4.2 (2q, 4H), 3.84-3.9 (m, 1H), 3.4-3.8 (m, 8H), 2.7-3.1 (m, 4H), 2.1-2.5 (m, 1H), 1.5-1.7 (m, 2H), 1.25-1.35 (2t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 21.63 ppm. MS: 680 (M + 1).

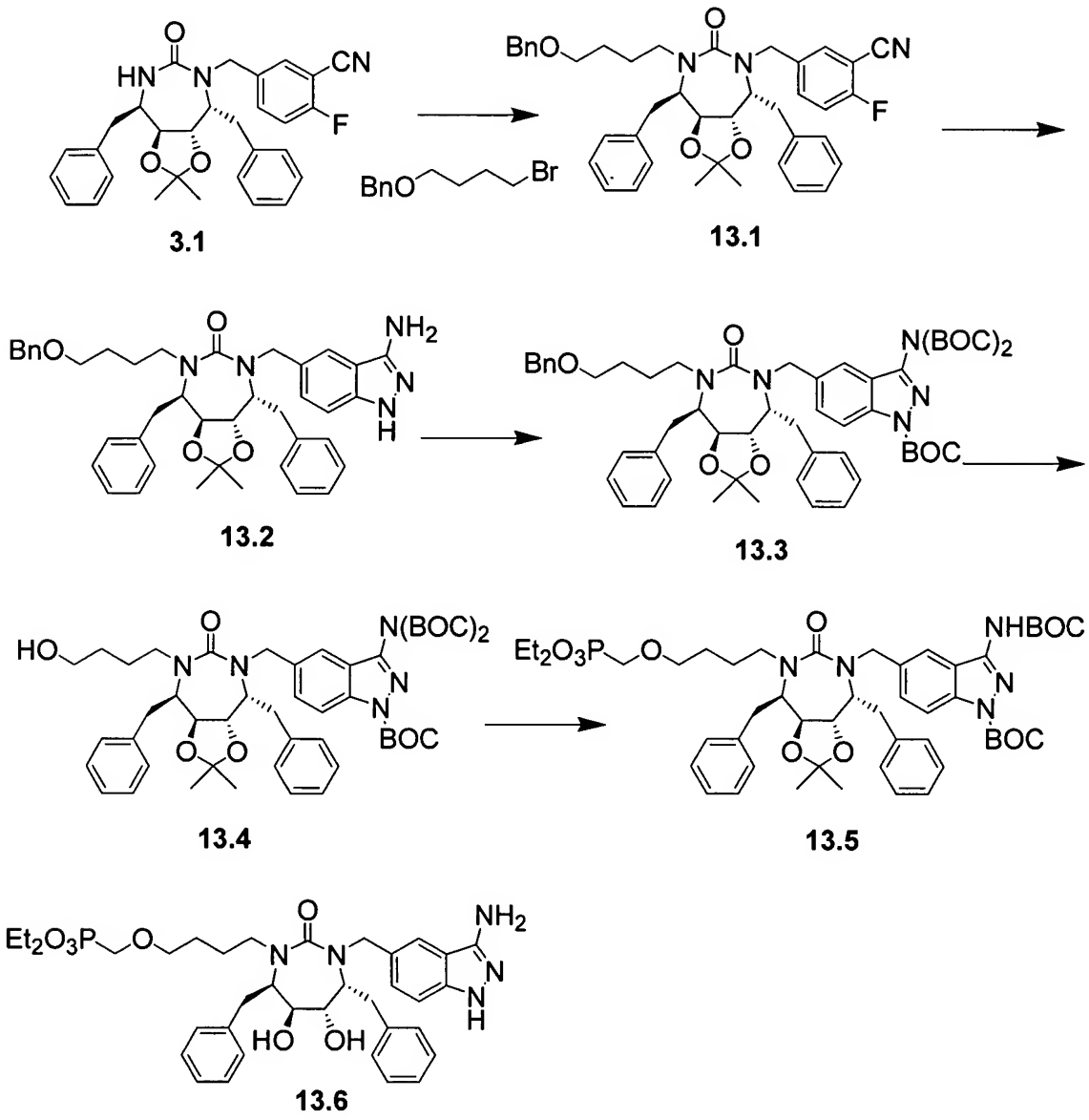
Scheme 12



Butyl lactate phosphonate 12.2: A pyridine solution (0.3 mL) of **11.9** (27 mg, 22 μmol), butyl lactate (31 mg, 265 μmol), and DCC (28 mg, 132 μmol) was stirred at room temperature for 5 min, then reacted at 70°C for 1.5 h. The reaction mixture was concentrated under reduced pressure, partitioned between methylene chloride and saturated NaCl solution, and purified by preparative TLC to give **12.1** (12 mg). A methylene chloride solution (0.8 mL) of **12.1** (12 mg) was treated with TFA (0.2 mL) for 4 h, concentrate. The residue was purified by preparative TLC to give **12.2** (3 mg, 16 %). NMR ($CDCl_3$ + ~10 %CD_3OD): δ 6.8-7.4 (m, 18H), 6.4-6.6 (m), 4.9-5.05 (m, 1H), 4.75 (d, 1H), 4.1-4.2 (m, 2H). 3.5-4.0 (m, 10H), 3.1-3.25 (m, 2H),

2.2-2.35 (m, 1H), 1.8-1.9 (m, 1H), 1.4 & 1.8 (m, 7H), 1.22 (t, 3H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 19.69 & 17.86 ppm.

Scheme 13



Benzyl ether 13.1: A DMF solution (5 mL) of **3.1** (1 g, 2 mmol) was treated with NaH (0.24 g of 60% oil dispersion, 6 mmol) for 30 min, followed by the addition of sodium iodide (0.3 g, 2 mmol),and benzoxybutyl bromide (0.58 g, 2.4 mmol). After the reaction for 5 h at room temperature, the reaction mixture was partitioned between methylene chloride and saturated NaCl, dried, and purified to give **13.1** (0.58 g, 44 %).

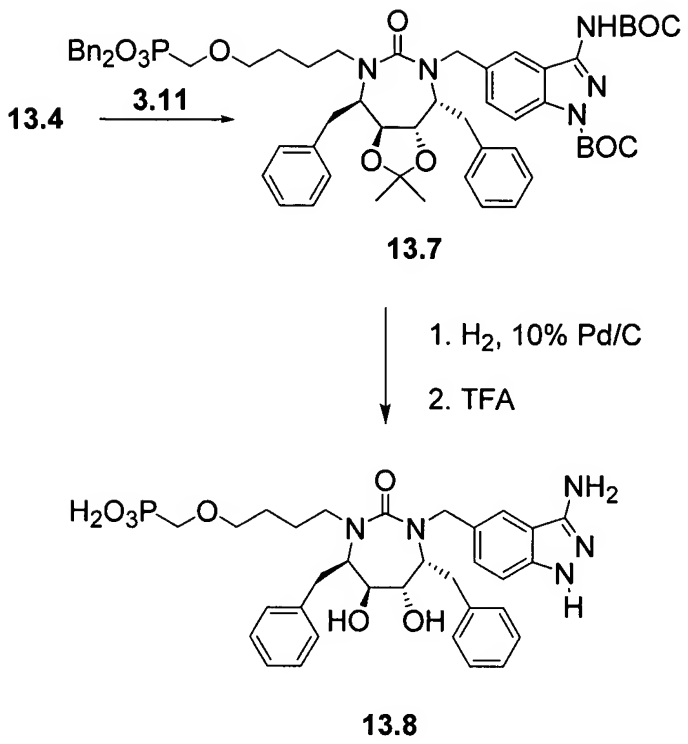
Aminoindazole 13.2: A n-butanol solution (10 mL) of **11.1** (0.58 g, 0.87 mmol) and hydrazine hydrate (0.88 g, 17.5 mmol) was heated at reflux for 4 h. The reaction mixture was concentrated under reduced pressure to give crude **13.2** (0.56 g).

Tri-BOC-aminoindazole 13.3: A methylene chloride solution (10 mL) of **13.2** (0.55 g, 0.82 mmol), DIPEA (0.42 g, 3.2 mmol), $(BOC)_2O$ (0.71 g, 3.2 mmol), and DMAP (0.3 g, 2.4 mmol) was stirred for 4 h at room temperature, partitioned between methylene chloride and 5% citric acid solution, dried, purified by silica gel chromatography to give **13.3** (0.56 g, 71 %, 2 steps).

3-Hydroxybutyl cyclic urea 13.4: An ethyl acetate/methanol solution (30 mL/5 mL) of **11.3** (0.55 g, 0.56 mmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (0.2 g) for 3 h. The catalyst was removed by filtration. The filtrate was concentrated under reduced pressure to afford crude **13.4** (0.5 g, 98 %).

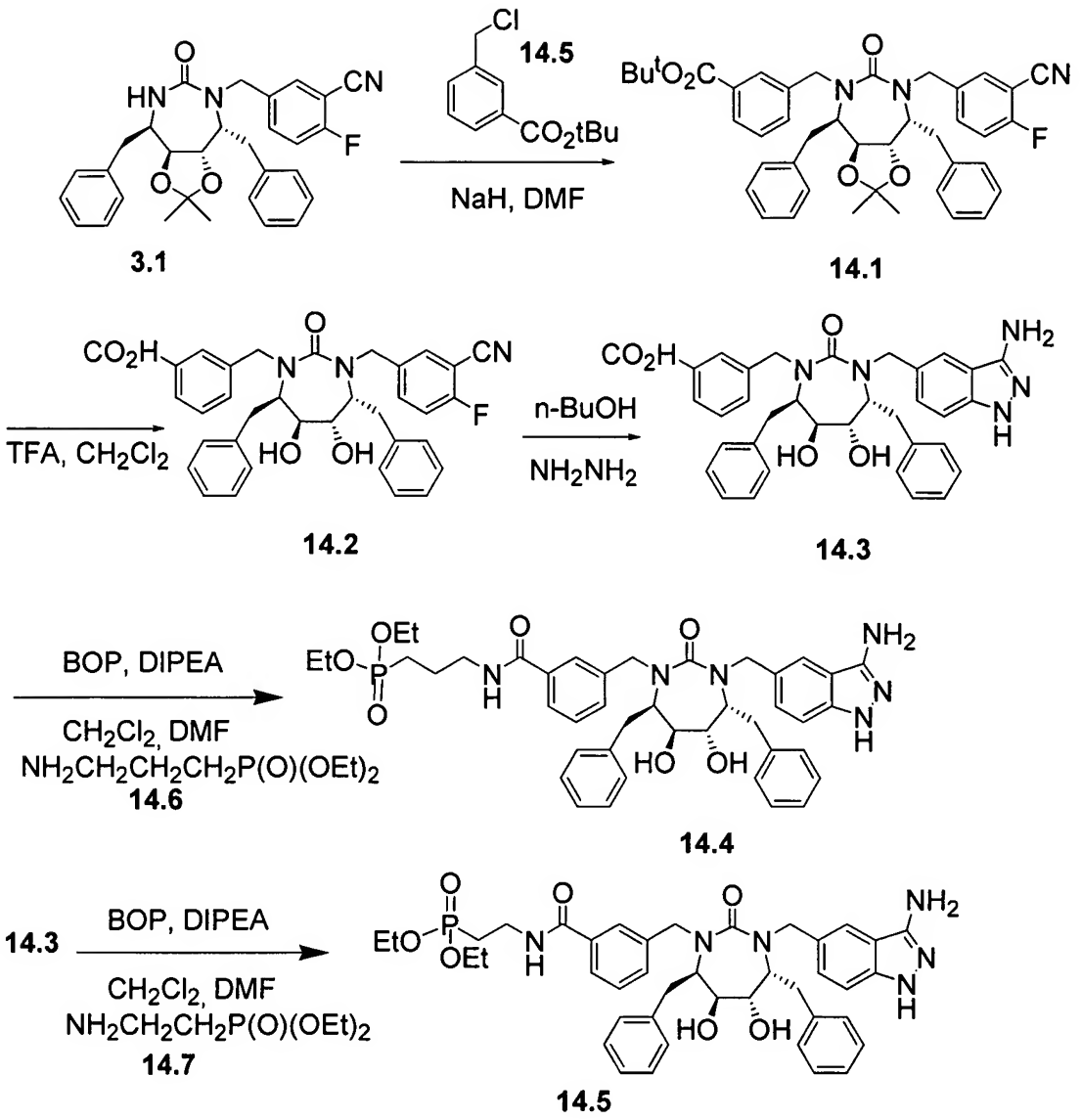
Diethyl phosphonate 13.6: A THF solution (1 mL) of **13.4** (5 mg, 56 μmol) and triflate diethyl phosphonate **5.3** (30 mg, 100 μmol) was cooled to –3°C, followed by addition of n-BuLi (80 μl of 2.5 M hexane solution, 200 μmol). After 2 h reaction, the reaction mixture was partitioned between methylene chloride and saturated NaCl solution, concentrated under reduced pressure to give crude **13.5**. The residue was dissolved in methylene chloride (0.8 mL) and treated with TFA (0.2 mL) for 4 h. concentrated under reduced pressure, and purified by HPLC to give **13.6** (8 mg, 21%). NMR ($CDCl_3$): δ 7.1-7.4 (m, 11H), 7.0-7.1 (m, 2H) 4.81 (d, 1H), 4.1-4.25 (m, 4H). 3.85-3.95 (m, 1H), 3.4-3.8 (m, 7H), 3.3-3.4 (m, 1H), 2.8 -3.25 (m, 5H), 2.0-2.15 (m, 1H), 1.3-1.85 (m, 10H). P NMR ($CDCl_3$): 21.45 ppm.

Scheme 13a



Phosphonic diacid 13.8: Compound **13.8** (4.5 mg) was prepared from **13.4** as described above for the preparation of **11.7** from **11.4** (Scheme 11). NMR (CD_3OD): δ 7.41 (s, 1H), 7.1-7.4 (m, 10H), 6.9-7.0 (m, 2H) 4.75 (d, 1H), 3.8-4.0 (m, 1H). 3.4-3.8 (m, 8H), 2.8 -3.25 (m, 5H), 2.1-2.25 (m, 1H), 1.6-1.85 (m, 4H). MS: 638 (M + 1).

Scheme 14



t-Butyl ester 14.1: A DMF solution (3 mL) of **3.1** (0.5 g, 1 mmol) was treated with NaH (80 mg of 60% oil dispersion, 2 mmol) for 10 min, followed by the addition of **14.5** (0.25 g, 1.1 mmol). After the reaction for 1 h at room temperature, the reaction mixture was partitioned between methylene chloride and saturated NaCl, dried, and purified to give **14.1** (0.4 g, 59%).

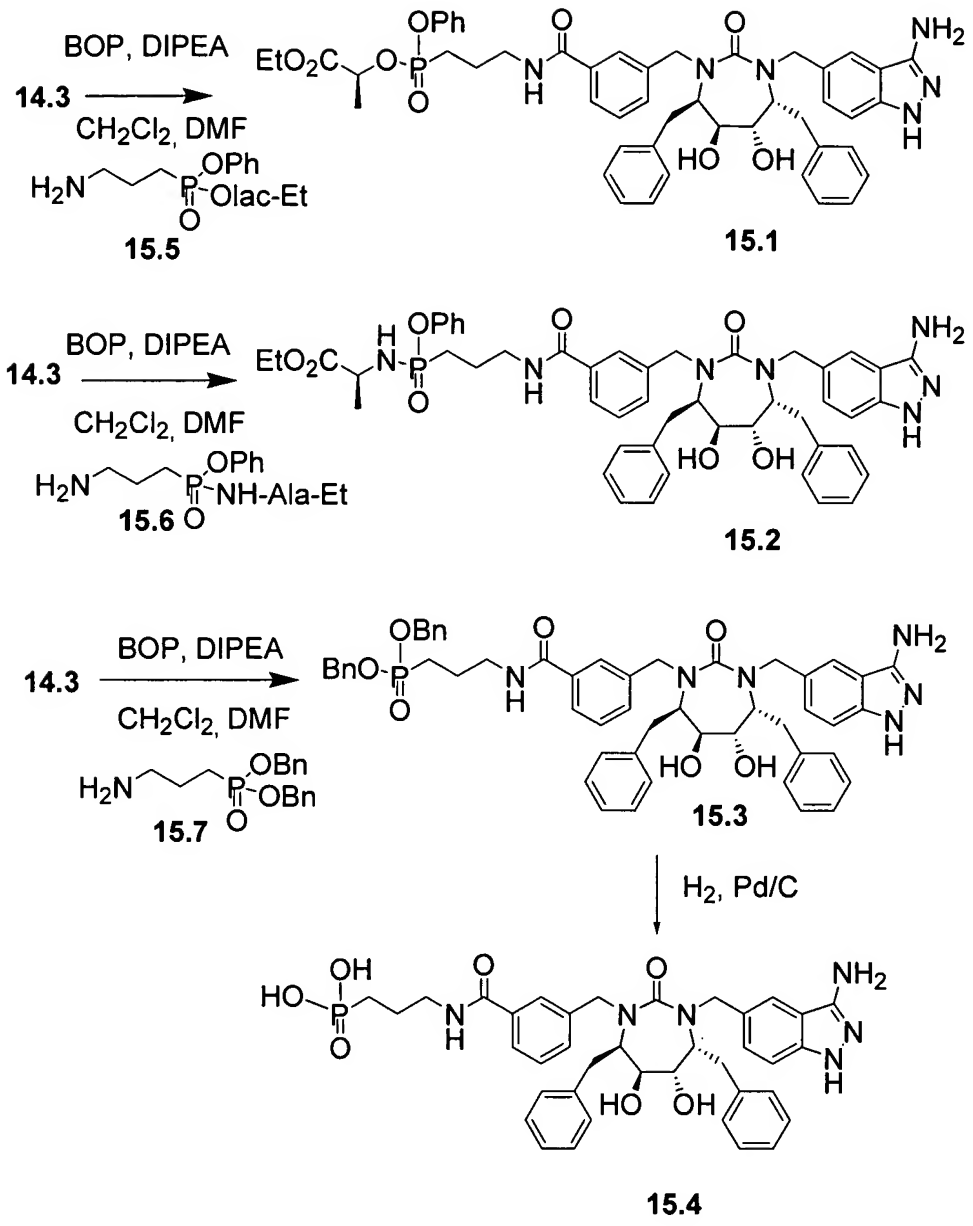
Aminoindazole derivative 14.3: A methylene chloride solution (5 mL) of **14.1** (0.4 g, 0.58 mmol) was treated with TFA (1 mL) at room temperature for 1.5 h, and then concentrated under reduced pressure to give crude **14.2**. The crude **14.2** was dissolved in n-BuOH (5 mL) and reacted with hydrazine hydrate (0.58 g, 11.6 mmol) at reflux for 5 h. The reaction mixture was

concentrated under reduced pressure and purified by silica gel chromatography to give the desired product **14.3** (0.37 g, quantitative yield).

Diethylphosphonate ester 14.4: A methylene chloride solution (3 mL) of **14.3** (23 mg, 38 μmol) was reacted with aminopropyl-diethylphosphonate **14.6** (58 mg, 190 μmol), DIPEA (50 mg, 380 μmol), and ByBOP (21 mg, 48 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by preparative TLC to give **14.4** (9 mg, 34 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.87 (t, 1H), 7.61 (b, 1H), 7.51 (s, 1H), 7.14-7.2 (m, 10 H), 6.93-7.0 (m, 4H), 4.79 (d, 2H), 3.99-4.04 (m, 4H), 3.38-3.65 (m, 6H), 2.60-3.2 (m, 6 H), 1.70-1.87 (m, 4H), 1.25 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 32.7 ppm.

Diethylphosphonate ester 14.5: A methylene chloride solution (2 mL) of **14.3** (13 mg, 21 μmol) was reacted with aminoethyl-diethylphosphonate oxalate **14.7** (23mg, 85 μmol), DIPEA (22 mg, 170 μmol), and ByBOP (12 mg, 25 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by preparative TLC to give 14.5 (5mg, 30%). Ms: 783 (M + 1). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.88 (b, 1H), 7.58 (b, 1H), 7.49 (s, 1H), 7.14-7.2 (m, 10 H), 6.90-7.0 (m, 4H), 4.75 (d, 2H), 3.90-4.04 (m, 4H), 2.50-3.3 (m, 6 H), 1.97-2.08 (m, 2H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 30.12 ppm.

Scheme 15



Monophenol-ethyl lactate phosphonate prodrug 15.1: A methylene chloride/DMF solution (2 mL/0.5 mL) of **14.3** (30 mg, 49 μmol) was reacted with aminopropyl-phenol-ethyl lactate phosphonate **15.5** (100 mg, 233 μmol), DIPEA (64 mg, 495 μmol), and BOP reagent (45 mg, 100 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by silica gel chromatography to give **15.1** (28 mg, 64 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.83 (b, 1H), 7.59 (b, 1H), 7.51 (s, 1H), 7.14-7.2 (m, 11 H), 6.90-7.0 (m, 4H), 4.75-4.87 (d + q, 3H), 4.10 (q, 2H),

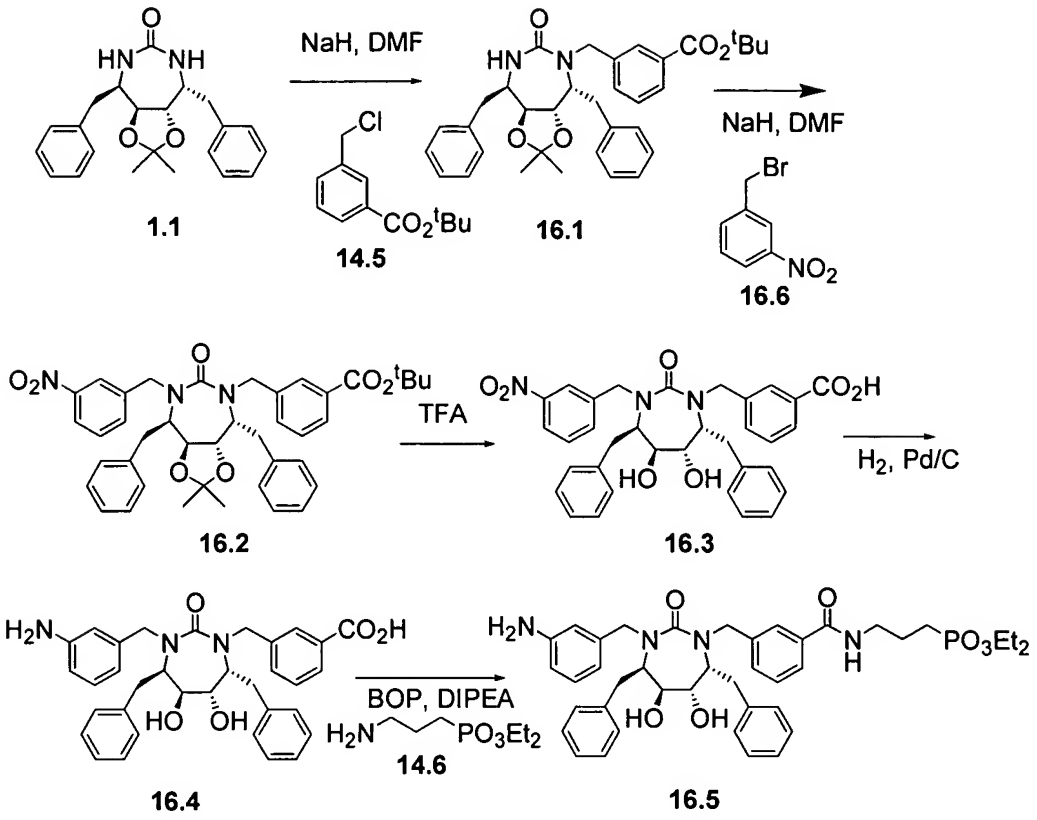
3.3-3.61 (m, 6H), 2.60-3.2 (m, 6H), 1.92-2.12 (m, 4H), 1.30 (d, 3H), 1.18 (t, 3H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 30.71 ppm. MS: 903 (M + 1).

Phenol-ethyl alanine phosphonate prodrug 15.2: A methylene chloride/DMF solution (2 mL/0.5 mL) of **14.3** (30 mg, 49 μmol) was reacted with aminopropyl-phenol-ethyl alanine phosphonate **15.6** (80 mg TFA salt, 186 μmol), DIPEA (64 mg, 500 μmol), and BOP reagent (45 mg, 100 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by preparative TLC to give **15.2** (12 mg, 27 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.91 (b, 1H), 7.61 (b, 1H), 7.52 (s, 1H), 7.14-7.2 (m, 11 H), 6.90-7.0 (m, 4H), 4.75 (d, 2H), 3.82-4.1 (2q, 3H), 3.4-3.65 (m, 6H), 2.60-3.15 (m, 6H), 1.8-2.0 (m, 4H), 1.3 (d, 3H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 32.98 & 33.38 ppm. MS: 902 (M + 1).

Dibenzyl phosphonate 15.3: A methylene chloride/DMF solution (2 mL/0.5 mL) of **14.3** (30 mg, 49 μmol) was reacted with aminopropyl dibenzyl phosphonate **15.7** (86 mg TFA salt, 200 μmol), DIPEA (64 mg, 500 μmol), and BOP reagent (45 mg, 100 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was triturated with methylene chloride/hexane. The solid was purified by preparative TLC to give **15.3** (20 mg, 44%). NMR ($CDCl_3$ + ~5%CD_3O): δ 7.50-7.58 (m, 2H), 7.14-7.3 (m, 21 H), 6.90-7.0 (m, 4H), 4.7-5.1 (m, 6H), 3.6-3.8 (m, 4H), 3.3-3.55 (m, 2H), 2.60-3.15 (m, 6H), 1.8-2.0 (m, 4H). P NMR ($CDCl_3$ + ~5 %CD_3OD): 33.7 ppm. MS: 907 (M + 1).

Phosphonic diacid 15.4: An ethanol solution (5 mL) of **15.3** (17 mg, 18.7 μmol) was hydrogenated at 1 atm in the presence of 10 % Pd/C for 4 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired product **15.4** (12 mg, 85%). NMR (CD_3O + 20%$CDCl_3$): δ 7.88 (b, 1H), 7.59 (b, 1H), 7.6 (s, 1H), 7.1-7.25 (m, 10 H), 6.90-7.1 (m, 4H), 4.8 (d, 2H + water peak), 3.6-3.8 (m, 4H), 3.4-3.5 (m, 2H), 1.85-2.0 (m, 4H).

Scheme 16



Monobenzyl derivative 16.1: A DMF solution (4 mL) of **1.1** (0.8 g, 2.2 mmol) was treated with NaH (0.18 g of 60% oil dispersion, 4.4 mmol) for 10 min at room temperature followed by the addition of 14.5 (0.5 g, 2.2 mmol). The resulting solution was reacted at room temperature for 2 h, worked up, and then purified to afford **16.1** (0.48 g, 40%).

3-Nitrobenzyl cyclic urea derivative 16.2: A DMF solution (0.5 mL) of **16.1** (65 mg, 117 μmol) was treated with NaH (15 mg of 60% oil dispersion, 375 μmol) for 10 min at room temperature, followed by the addition of 3-nitrobenzyl bromide (33 mg, 152 μmol). The resulting solution was reacted at room temperature for 1 h, worked up, and purified by preparative TLC to afford **16.2** (66 mg, 82%).

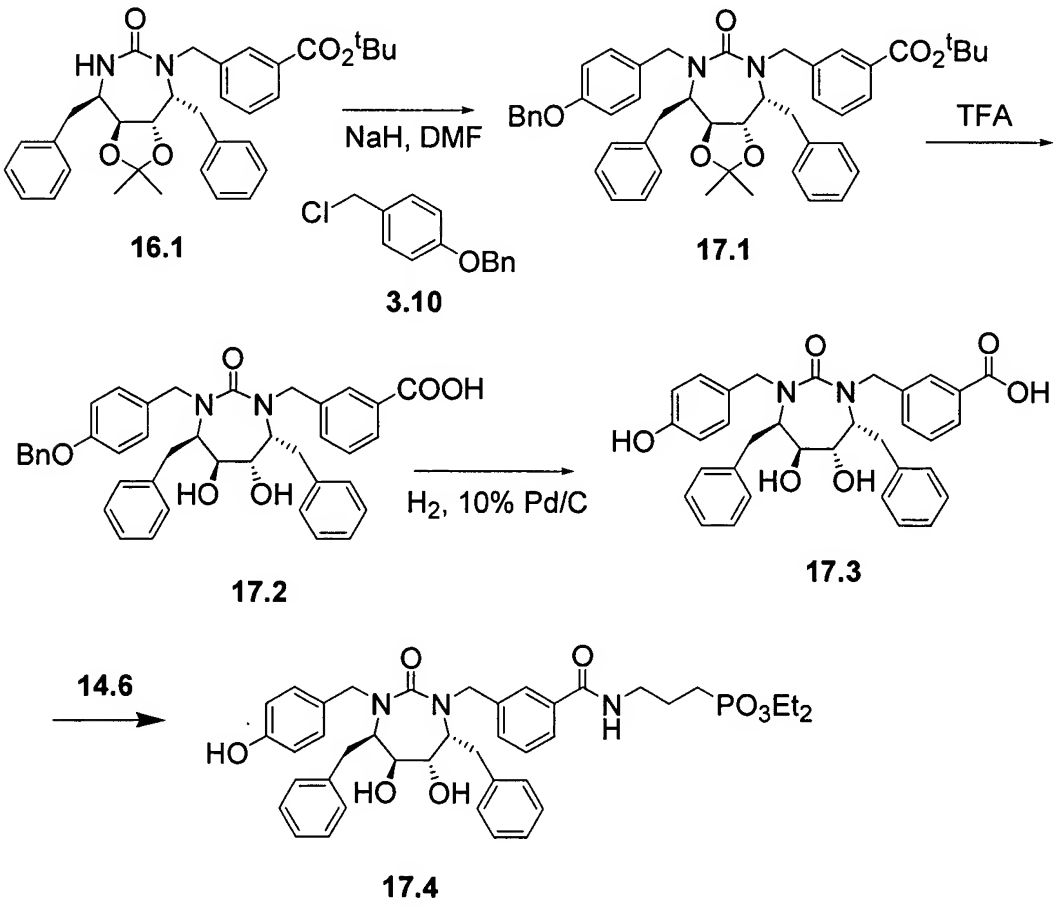
Diol 16.3: A methylene chloride solution (2 mL) of **16.2** (46 mg, 61 μmol) was treated with TFA (0.4 mL) for 2 h at room temperature, and then concentrated under reduced pressure to afford **16.3**. This material was used without further purification.

3-Aminobenzyl cyclic urea 16.4: An ethyl acetate/ethanol (5 mL/1 mL) solution of **16.3** (crude) was hydrogenated at 1 atm in the presence of 10% Pd/C for 2 h. The catalyst was

removed by filtration. The filtrate was concentrated under reduced pressure, and purified by preparative TLC to afford **16.4** (26 mg, 70%, 2 steps).

Diethyl phosphonate 16.5: A methylene chloride/DMF solution (2 mL/0.5 mL) of **16.4** (24 mg, 42 μmol) was reacted with aminopropyl-diethylphosphonate ester TFA salt **14.6** (39 mg, 127 μmol), DIPEA (27 mg, 210 μmol), and BOP reagent (28 mg, 63 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was purified by preparative TLC to give **16.5** (20.7 mg, 63 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.62 (b, 1H), 7.51 (s, 1H), 7.0-7.35 (m, 12 H), 6.95 (d, 2H), 6.85 (d, 2H), 4.6-4.71 (2d, 2H), 3.95-4.1 (m, 4H). 3.3-3.55 (m, 3H), 2.60-2.8 (m, 2H), 2.95-.3. 15 (m, 4 H), 1.85-2.0 (m, 4H), 1.25 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 32.65 ppm.

Scheme 17



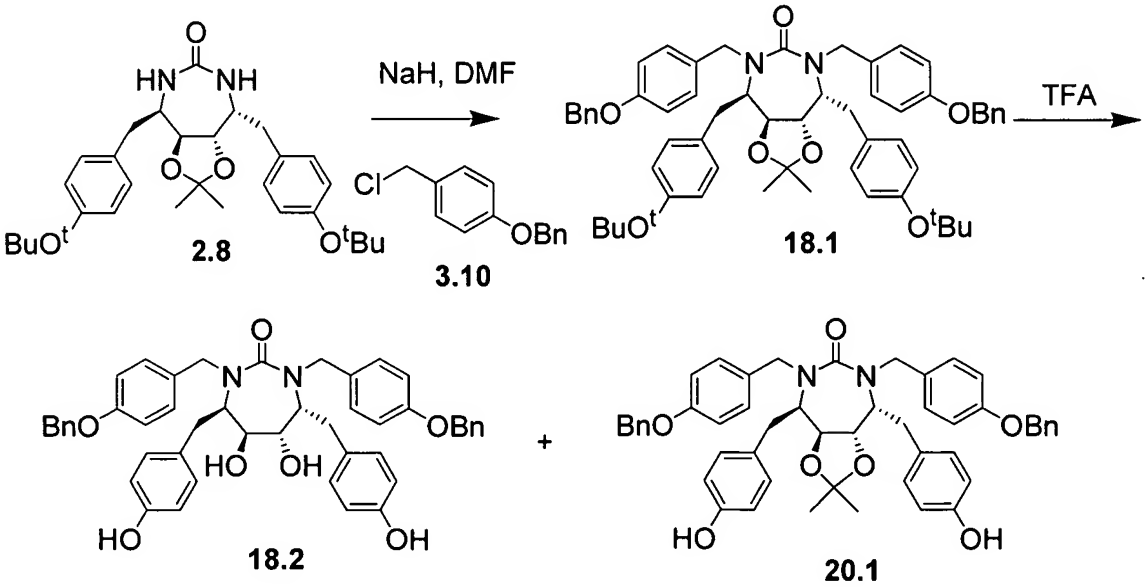
p-Benzoxybenzyl cyclic urea derivative 17.1: A DMF solution (0.5 mL) of **16.1** (65 mg, 117 μmol) was treated with NaH (15 mg of 60% oil dispersion, 375 μmol) for 10 min at

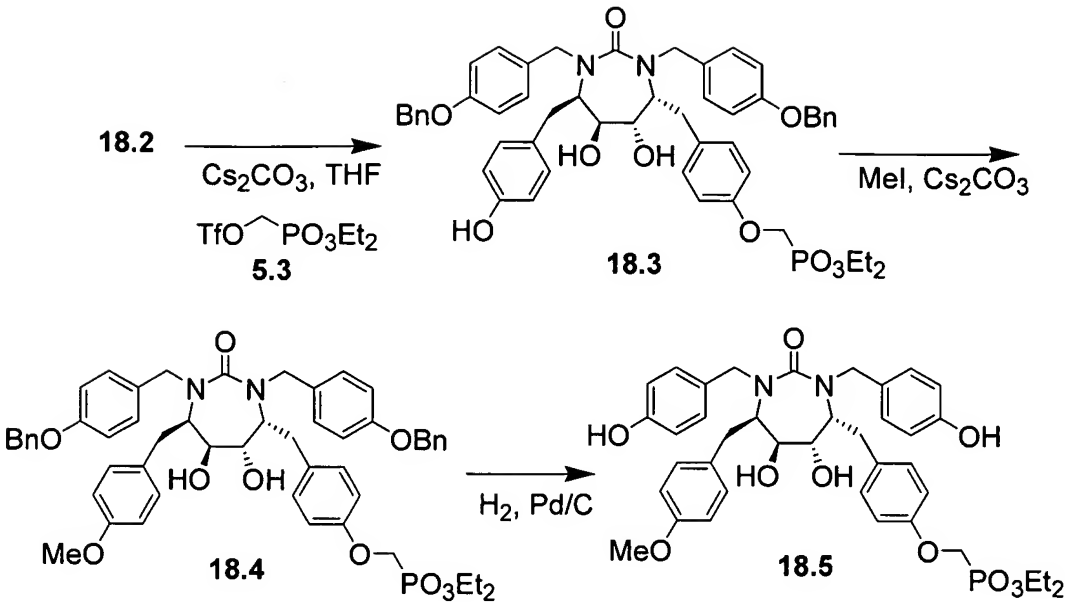
room temperature, followed by the addition of 4-benzoxy benzyl chloride **3.10** (35 mg, μmol). The resulting solution was stirred for 2 h at room temperature. The reaction mixture was concentrated under reduced pressure, purified by preparative TLC to generate **17.1** (62 mg, 70%).

Diethyl phosphonate 17.3: A methylene chloride solution (2 mL) of **17.1** (46 mg, 61 μmol) was treated with TFA (0.4 mL) for 2 h at room temperature, and then concentrated under reduced pressure to give crude **17.2**. An ethyl acetate/ethanol solution (3 mL/2 mL) of the crude **17.2** was then hydrogenated at 1 atm in the presence of 10% Pd/C (10 mg) for 5 h at room temperature. The catalyst was removed by filtration. The filtrate was concentrated under reduced pressure to afford **17.3** (crude).

Diethyl phosphonate cyclic urea 17.4: A methylene chloride/DMF solution (2 mL/0.5 mL) of **17.3** (25 mg, 42 μmol) was reacted with aminopropyl-diethylphosphonate ester TFA salt **14.6** (40 mg, 127 μmol), DIPEA (27 mg, 210 μmol), and BOP reagent (28 mg, 63 μmol) at room temperature for 2 h, and then concentrated under reduced pressure. The residue was purified by preparative TLC to give **17.4** (14.6 mg, 44 %). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.82 (t), 7.62 (d, 1H), 7.51 (s, 1H), 7.05-7.35 (m, 10 H), 6.8-6.95 (2d, 4H), 6.85 (d, 2H), 4.8 (d, 1H), 4.65 (d, 1H), 3.95-4.1 (m, 4H). 3.4-3.75 (m, 6H), 2.60-3.2 (m), 1.85-2.0 (m, 4H), 1.25 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 32.72 ppm.

Scheme 18





Dibenzyl derivative 18.1: A DMF solution (3 mL) of compound **2.8** (0.4 g, 0.78 mmol) was reacted with 60%NaH (0.13 g, 1.96 mmol), 4-benzoxy benzylchloride **3.10** (0.46 g, 1.96 mmol) and sodium iodide (60 mg, 0.39 mmol) at room temperature for 4 h. The reaction mixture was partitioned between methylene chloride and saturated $NaHCO_3$ solution. The organic phase was isolated, dried over Na_2SO_4, concentrated under reduced pressure, and purified by silica gel chromatography to give the desired product **18.1** (0.57 g, 81%).

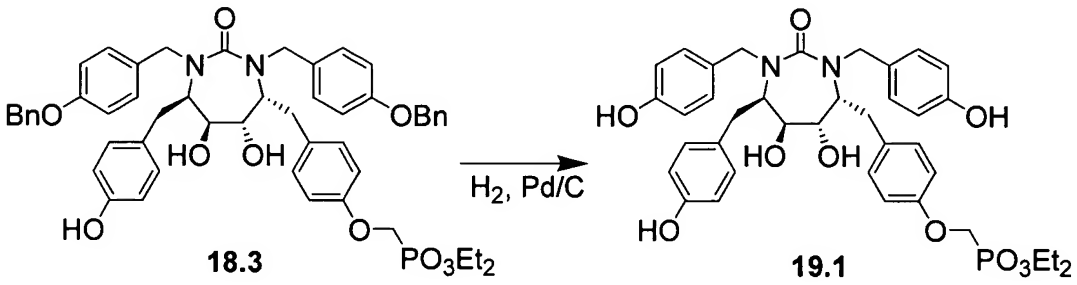
Diol derivative 18.2 and diphenol derivative 20.1: A methylene chloride solution (4 mL) of **18.1** (0.57 g, 0.63 mmol) was treated with TFA (1 mL) at room temperature for 20 min, concentrated under reduced pressure, and purified by silica gel chromatography to give diol derivative **18.2** (133 mg, 28 %) and diphenol derivative **20.1** (288 mg. 57.6%).

Monophosphonate derivative 18.3: A THF solution (10 mL) of **18.2** (130 mg, 0.17 mmol) was stirred with cesium carbonate (70 mg, 0.21 mmol) and diethylphosphonate triflate **5.3** (52 mg, , 0.17 mmol) at room temperature for 4 h.. The reaction mixture was concentrated under reduced pressure and purified to give **18.3** (64 mg, 41 %), and recovered **18.2** (25 mg, 19%).

Methoxy derivative 18.4: A THF solution (2 mL) of **18.3** (28 mg, 25 μmol) was treated with cesium carbonate (25 mg, 76 μmol) and iodomethane (10 eq. Excess) at room temperature for 5 h. The reaction mixture was concentrated under reduced pressure and partitioned between methylene chloride and saturated $NaHCO_3$. The organic phase was separated, concentrated under reduced pressure and the residue purified by preparative TLC to afford **18.4** (22 mg, 78%).

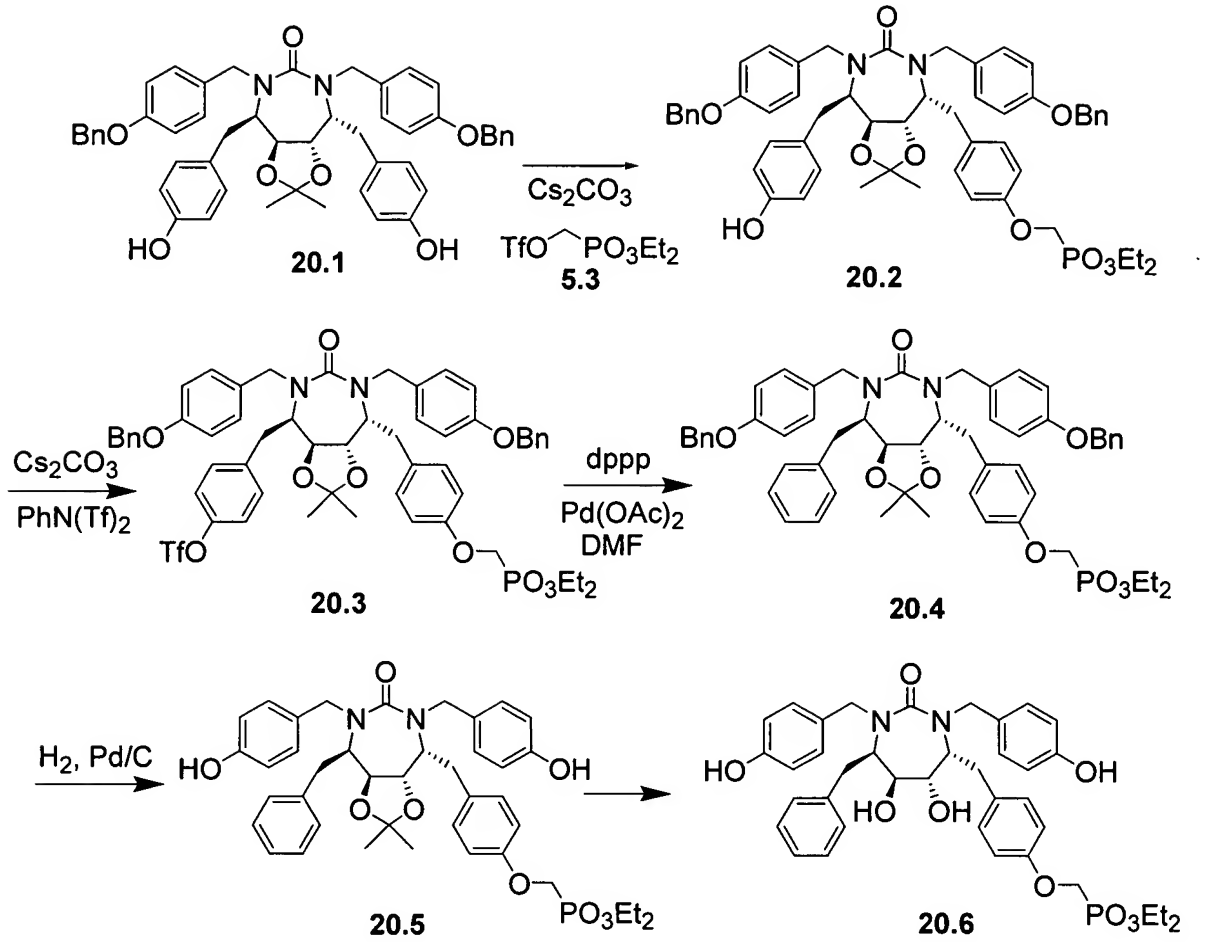
Diethylphosphonate 18.5: An ethyl acetate/ethanol (2 mL/2 mL) solution of **18.4** (22 mg, 24 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C for 3 h. The catalyst was removed by filtration, the filtrate was concentrated under reduced pressure to give the desired product **18.5** (18 mg, quantitative). NMR ($CDCl_3$ + ~10 %CD_3O): δ 6.7-7.0 (m, 12 H), 6.62-6.69 (m, 4H), 4.65 (d, 1H), 4.50 (d, 1H), 4.18-4.3 (m, 6H). 3.75 (s, 3H), 3.3-3.4 (m, 4H), 2.8-3.0 (m, 6H), 1.30 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 20.16 ppm.

Scheme 19



Diethyl phosphonate 19.1: An ethyl acetate/ethanol (2 mL/1 mL) solution of **18.3** (14 mg, 15.5 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (5 mg) for 3 h. The catalyst was then removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired product **19.1** (10 mg, 90%). NMR ($CDCl_3$ + ~15 %CD_3O): δ 6.6-7.0 (m, 16 H), 4.5-4.65 (2d, 2H), 4.1-4.3 (m, 6H). 2.7-3.0 (m, 6H), 1.29 (t, 6H). P NMR ($CDCl_3$ + ~15 %CD_3OD): 20.12 ppm.

Scheme 20



Monophosphonate 20.2: A THF solution (8 mL) of **20.1** (280 mg, 0.36 mmol) was stirred with cesium carbonate (140 mg, 0.43 mmol) and diethylphosphonate triflate **5.3** (110 mg, 0.36 mmol) at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and purified to give **20.2** (130mg, 39%), and recovered **20.1** (76 mg, 27%).

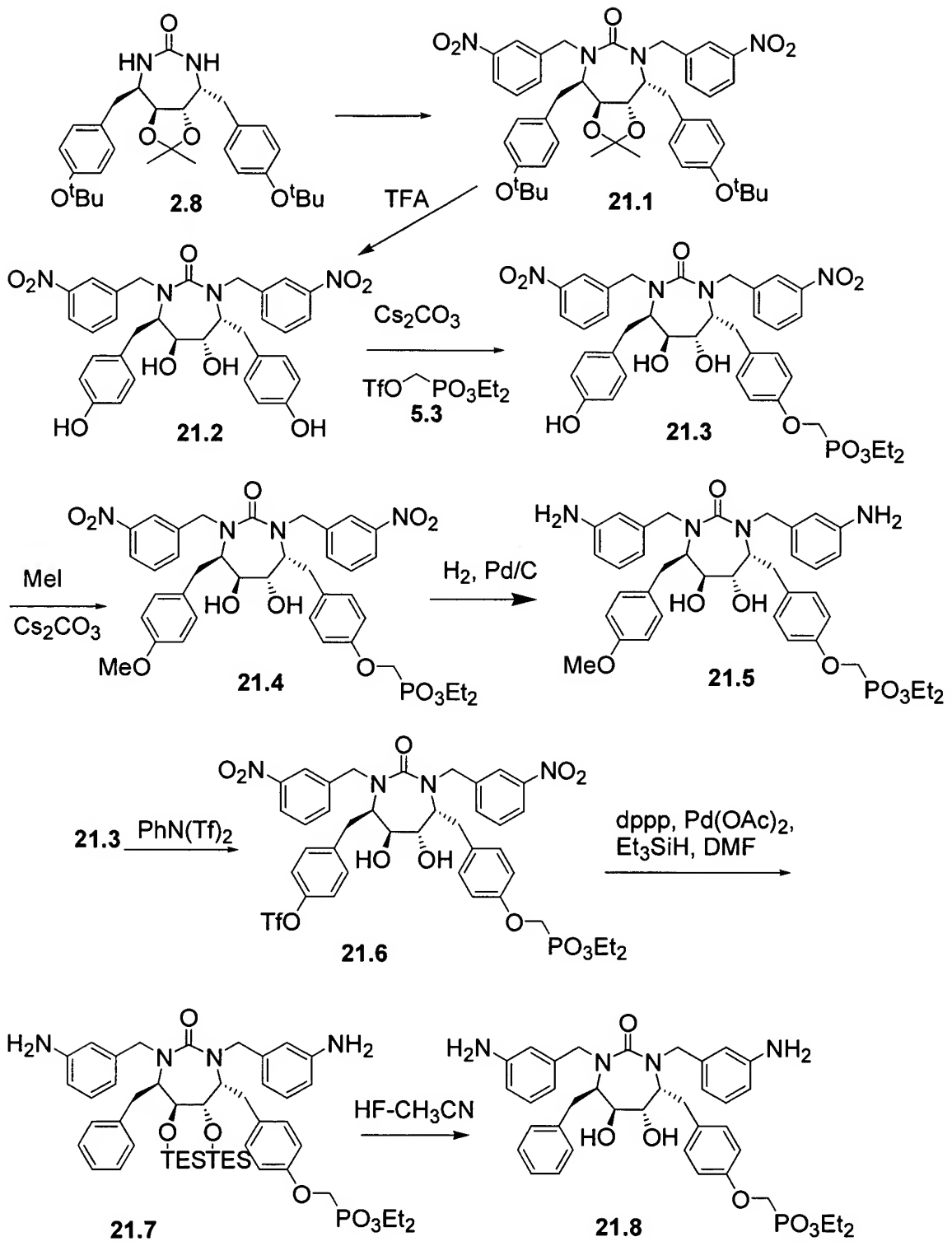
Triflate derivative 20.3: A THF solution (6 mL) of **20.2** (130 mg, 0.13 mmol) was stirred with cesium carbonate (67 mg, 0.21 mmol) and N-phenyltrifluoromethane-sulfonimide (60mg, 0.17 mmol) at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and purified to give **20.3** (125 mg, 84%).

Benzyl ether 20.4: To a DMF solution (2 mL) of $Pd(OAc)_2$ (60 mg, 267 μmol), and dppp (105 mg. 254 μmol) was added **20.3** (120 mg, 111 μmol) under nitrogen, followed by the addition of triethylsilane (0.3 mL). The resulting solution was stirred at room temperature for 4

h, then concentrated under reduced pressure. The residue was purified by silica gel chromatography to afford **20.4** (94 mg, 92%).

Diethyl phosphonate 20.6: An ethyl acetate/ethanol (2 mL/2 mL) solution of **20.4** (28 mg, 30 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (5 mg) for 3 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired product **20.5**. The crude product **20. 5** was redissolved in methylene chloride (2 mL) and treated with TFA (0.4 mL) and a drop of water. After 1 h stirring at room temperature, the reaction mixture was concentrated under reduced pressure, and purified by preparative TLC plate to give **20.6** (18 mg, 85 %, 2 steps). δ 6.6-7.3 (m, 17 H), 4.65 (d, 1H), 4.58 (d, 1H), 4.18-4.3 (m, 6H), 3.3-3.5 (m, 4H), 2.8-3.1 (m), 1.34 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 20.16 ppm. MS: 705 (M + 1).

Scheme 21



Bis-(3-nitrobenzyl) derivative 21.1: A DMF solution (2 mL) of compound **2.8** (0.3 g, 0.59 mmol) was reacted with 60%NaH (0.07 g, 1.76 mmol), 3-nitrobenzyl bromide (0.38 g, 1.76

mmol) and sodium iodide (60 mg, 0.39 mmol) at room temperature for 3 h. The reaction mixture was partitioned between methylene chloride and saturated $NaHCO_3$ solution. The organic phase was isolated, dried over Na_2SO_4, concentrated under reduced pressure, and purified by silica gel chromatography to give the desired product **21.1** (0.37 g, 82%).

Diphenol derivative 21.2: A methylene chloride solution (4 mL) of **21.1** (0.37 g, 0.47 mmol) was treated with TFA (1 mL) at room temperature for 3 h, and then concentrated under reduced pressure, and azeotroped with CH_3CN twice to give diphenol derivative **21.2** (0.3 g, quantitative).

Monophosphonate derivative 21.3: A THF solution (8 mL) of **18.2** (0.28g, 0.44 mmol) was stirred with cesium carbonate (0.17 g, 0.53 mmol) and diethylphosphonate triflate **5.3** (0.14 g, 0.44 mmol) at room temperature for 4 h. The reaction mixture was concentrated under reduced pressure and purified to give **21.3** (120 mg, 35%), and recovered **21.2** (150 mg, 53%).

Methoxy derivative 21.4: A THF solution (2 mL) of **21.3** (9 mg, 11 μmol) was treated with cesium carbonate (15 mg, 46 μmol) and iodomethane (10 eq. Excess) at room temperature for 6 h. The reaction mixture was concentrated under reduced pressure and partitioned between methylene chloride and saturated $NaHCO_3$. The organic phase was separated, dried over sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by preparative TLC to afford **21.4** (9 mg).

Diethylphosphonate 21.5: A ethyl acetate/ethanol (2 mL/0.5 mL) solution of **21.4** (9 mg, 11 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C for 4 h. The catalyst was removed by filtration, and the filtrate was concentrated under reduced pressure to give the desired product 21.5 (4.3 mg, 49%, 2 steps). NMR ($CDCl_3$ + ~10 %CD_3O): δ 7.0-7.10 (m, 6 H), 6.8-6.95 (m, 4H), 6.5-6.6 (m, 4H), 6.4-6.45 (m, 2H), 4.72 (d, 2H), 4.18-4.3 (m, 6H). 3.72 (s, 3H), 3.4-3.5 (m, 4H), 2.8-3.0 (m, 6H), 1.34 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 19.93 ppm.

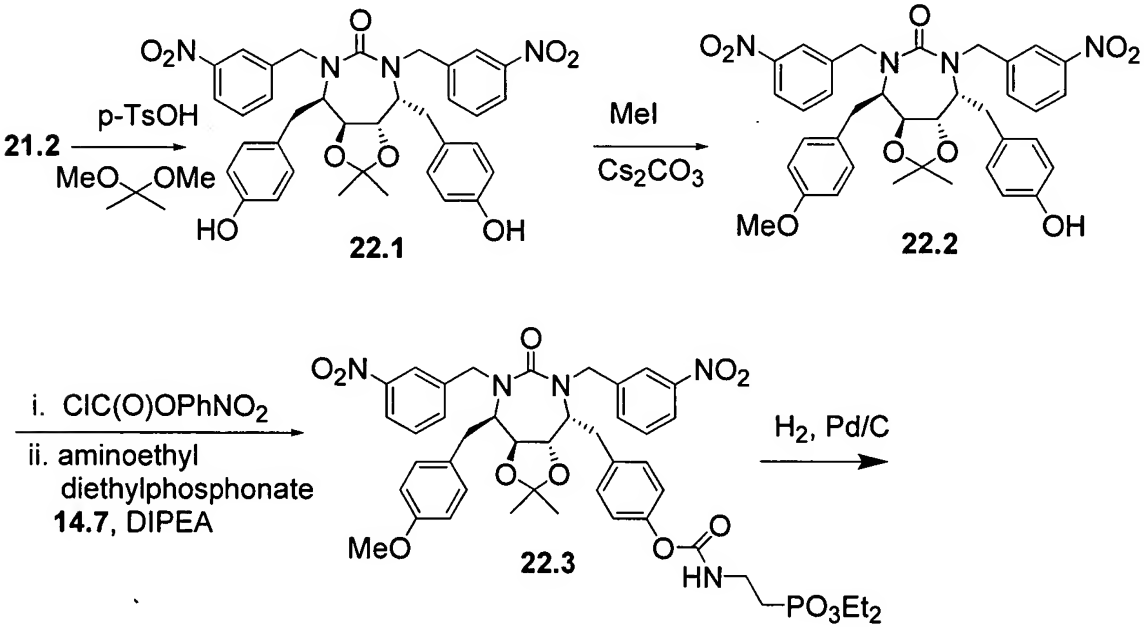
Triflate 21.6: A THF solution (6 mL) of **21.3** (0.1g, 0.14 mmol), cesium carbonate (0.07 g, 0.21 mmol), and N-phenyltrifluoromethane-sulfonimide (60mg, 0.17 mmol) was stirred at room temperature for 4 h, and then concentrated under reduced pressure, and worked up. The residue was purified by silica gel chromatography to give **21.6** (116 mg, 90%).

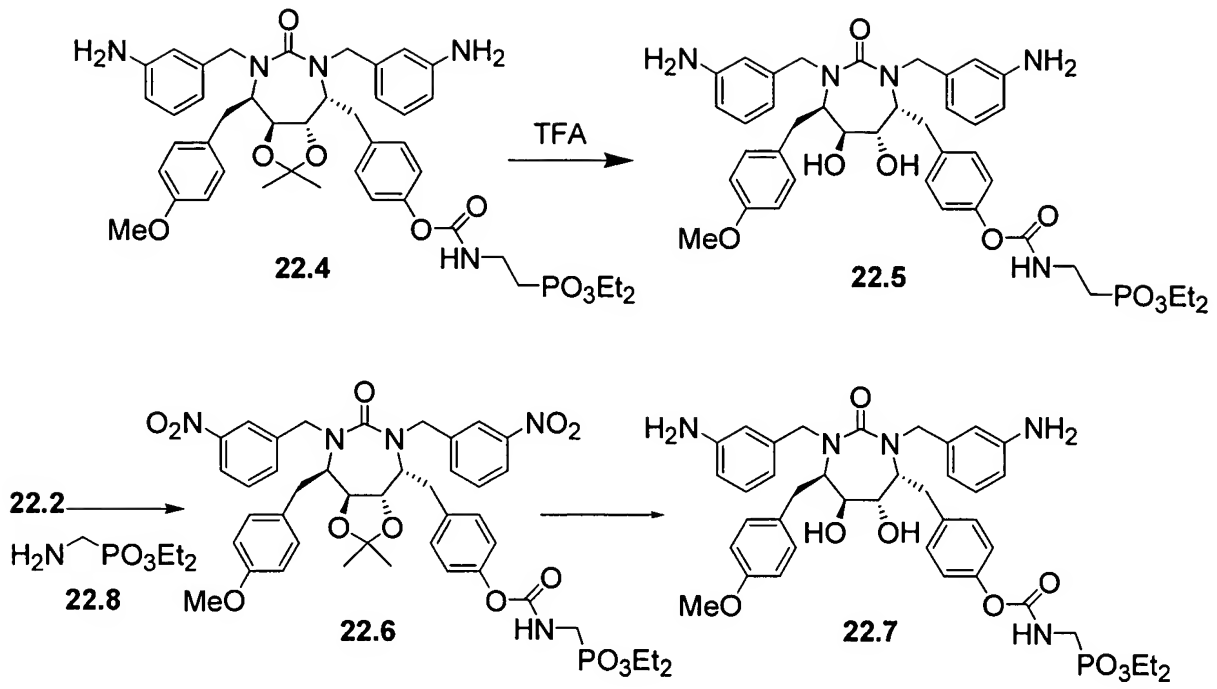
Diamine 21.7: A DMF solution (2 mL) of **21.6** (116 mg, 127 μmol), dppp (60 mg, 145 μmol), and $Pd(OAc)_2$ (30 mg, 134 μmol) was stirred under nitrogen, followed by addition of

triethylsilane (0.3 mL), and reacted for 4 h at room temperature. The reaction mixture was worked up and purified to give **21.7** (50 mg).

Diethyl phosphonate 21.8: An acetonitrile solution (1 mL) of crude **21.7** (50 mg) was treated with 48% HF (0.1 mL) for 4 h. The reaction mixture was concentrated under reduced pressure, and purified to give **21.8** (10 mg, 11% (2 steps). NMR ($CDCl_3$ + ~10%CD_3O): δ 7.05-7.30 (m, 9 H), 6.8-6.95 (d, 2H), 6.4-6.6 (m, 6H), 4.72 (d, 2H), 4.18-4.3 (m, 6H). 3.4-3.5 (m, 4H), 2.8-3.0 (m, 6H), 1.34 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 19.83 ppm.

Scheme 22





Acetonide 22.1: An acetone/2,2-diemethoxypropane solution (15 mL/5 mL) of compound **21.2** (240 mg, 0.38 mmol) and pyridinium toluenesulfonate (10 mg) was heated at reflux for 30 min. After cooled to room temperature, the reaction mixture was concentrated under reduced pressure. The residue was partitioned between methylene chloride and saturated $NaHCO_3$ aqueous solution, dried, concentrated under reduced pressure and purified to afford **22.1** (225 mg, 88%).

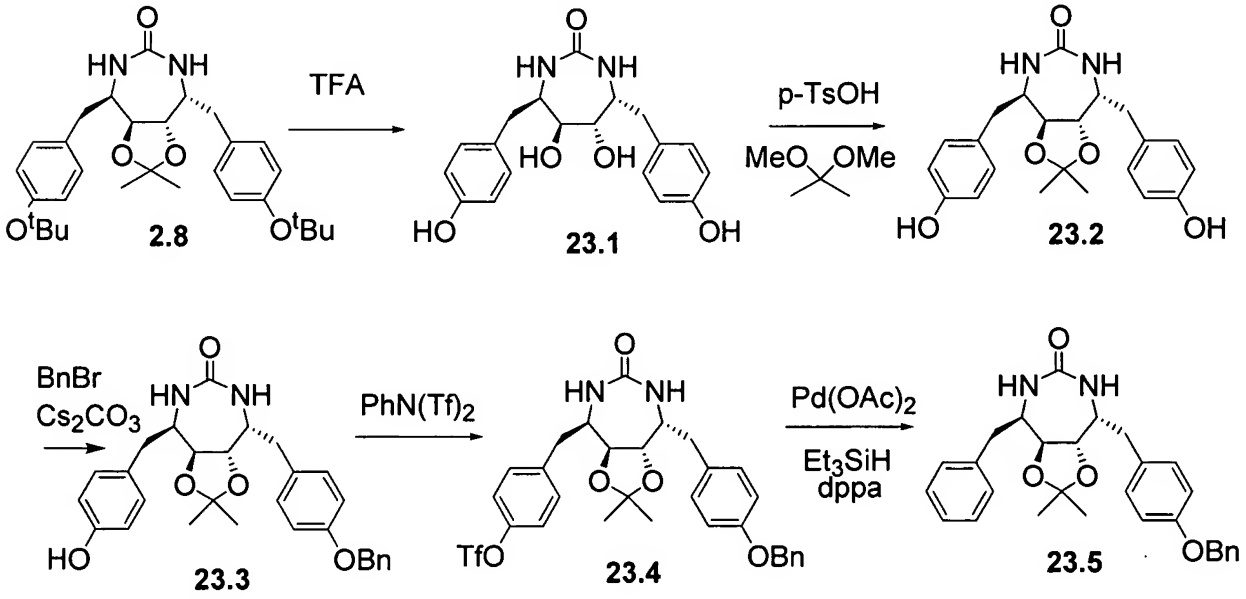
Monomethoxy derivative 22.2: A THF solution (10 mL) of **22.1** (225 mg, 0.33 mmol) was treated with cesium carbonate (160 mg, 0.5 mmol) and iodomethane (52 mg. 0.37 mmol) at room temperature overnight. The reaction mixture was concentrated under reduced pressure, and purified by preparative silica gel column chomatography to afford **22.2** (66 mg, 29%) and recovered starting material **22.1** (25 mg, 11%).

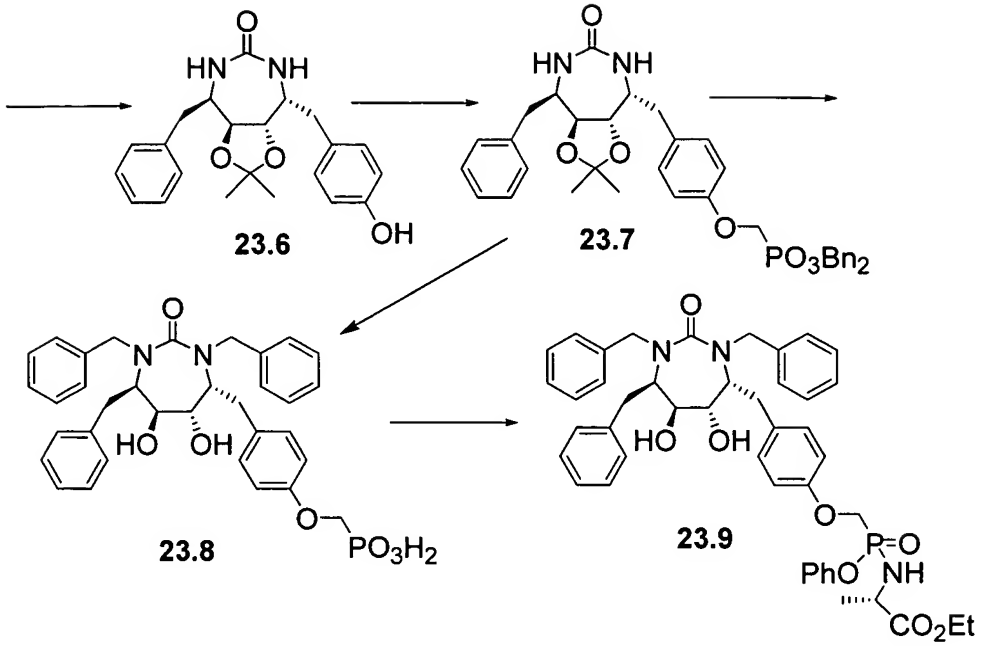
Diethyl phosphonate 22.3: A methylene chloride solution (2 mL) of **22.2** (22 mg, 32 μmol), DIPEA (9 mg, 66 μmol), and p-nitrophenyl chloroformate (8 mg, 40 μmol) was stirred at room temperature for 30 min. The resulting reaction mixture was reacted with DIPEA (10 mg, 77 μmol), and aminoethyl diethylphosphonate **14.7** (12 mg. 45 μmol) at room temperature overnight. The reaction mixture was washed with 5% citric acid solution, saturated $NaHCO_3$, dried, and purified by preparative TLC to afford **22.3** (12 mg, 43%).

Bis(3-aminobenzyl)-diethylphosphonate ester 22.5: An ethyl acetate/t-BuOH (4 mL/2 mL) solution of **22.3** (12 mg, 13 μmol) was hydrogenated at 1 atm in the presence of 10% Pd/C 95 mg) at room temperature for 5 h. The catalyst was removed by filtration. The filtrate was concentrated under reduced pressure, and purified by preparative TLC to give **22.4** (8 mg, 72%). A methylene chloride solution (0.5 mL) of **22.4** (8 mg) was treated with TFA (0.1 mL) at room temperature for 1 h., concentrated under reduced pressure, and then azeotroped with CH_3CN twice to afford **22.5** (8.1 mg, 81%). NMR ($CDCl_3$ + ~10 %CD_3OD): δ 7.2 (d, 1H), 6.95-7.15 (m, 6H), 6.75-6.9 (m, 5 H), 4.66 (d, 1H), 4.46 (d, 1H), 4.06-4.15 (m, 4H). 3.75 (s, 3H), 3.6-3.7 (m, 4H), 2.6-3.1 (m, 6H), 2.0-2.1 (m, 2H), 1.30 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 29.53 ppm. MS: 790 (M + 1).

Bis(3-aminobenzyl) diethylphosphonate ester 22.7: Compound **22.7** was prepared from **22.2** (22 mg, 32 μmol) and aminomethyl diethylphosphonate **22.8** as shown above for the preparation of **22.5** from **22.2.** NMR ($CDCl_3$ + ~10 %CD_3OD): δ 7.24 (d, 1H), 6.8-7.12 (m, 11H), 4.66 (d, 1H), 4.45 (d, 1H), 4.06-4.15 (m, 4H). 3.75 (s, 3H), 2.6-3.1 (m, 6H), 1.30 (t, 6H). P NMR ($CDCl_3$ + ~10 %CD_3OD): 22.75 ppm. MS: 776 (M + 1).

Scheme 23





Diol 23.1: To a solution of compound **2.8** (2.98 g, 5.84 mmol) in methylene chloride (14 mL) was added TFA (6 mL). The resulted mixture was stirred at room temperature for 2 h. Methanol (5 mL) and additional TFA (5 mL) were added. The reaction mixture was stirred for additional 4 h and then concentrated under reduced pressure. The residue was washed with hexane/ethyl acetate (1:1) and dried to afford compound **23.1** (1.8 g, 86%) as an off-white solid.

Benzyl ether 23.3: To a solution of compound **23.1** (1.8 g, 5.03 mmol) in DMF (6 mL) and 2,2-dimethoxyl propane (12 mL) was added p-toluenesulfonic acid monohydrate (0.095 g, 0.5 mmol). The resultant mixture was stirred at 65°C for 3 h. The excess 2,2-dimethoxyl propane was slowly distilled. The reaction mixture was cooled to room temperature and charged with THF (50 mL), benzyl bromide (0.8 mL, 6.73 mmol) and cesium carbonate (2.0 g, 6.13 mmol). The resulted mixture was stirred at 65°C for 16 h. The reaction was quenched with acetic acid aqueous solution (4%, 100 mL) at 0°C, and extracted with ethyl acetate. The organic phase was dried over magnesium sulfate and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford desired mono protected compound **23.3** (1.21 g, 49%).

Benzyl ether 23.5: To a solution of compound **23.3** (0.65 g, 1.33 mmol) and N-phenyltrifluoromethanesulfonimide (0.715 g, 2 mmol) in THF (12 mL) was added cesium carbonate (0.65 g, 2 mmol). The mixture was stirred at room temperature for 3 h. The reaction mixture was filtered through a pad of silica gel and concentrated under reduced pressure. The

residue was purified on silica gel chromatography to give triflate **23.4** (0.85 g). To a solution of 1,3-bis(diphenylphosphino)propane (0.275g, 0.66 mmol) in DMF (10 mL) was added palladium(II) acetate (0.15 g, 0.66 mmol) under argon. This mixture was stirred for 2 min. and then added to triflate **23.4**. After stirring for 2 min., triethylsilane was added and the resulted mixture was stirred for 1.5 h. The solvent was removed under reduced pressure and the residue was purified by chromatography on silica gel to afford compound **23.5** (0.56 g, 89%).

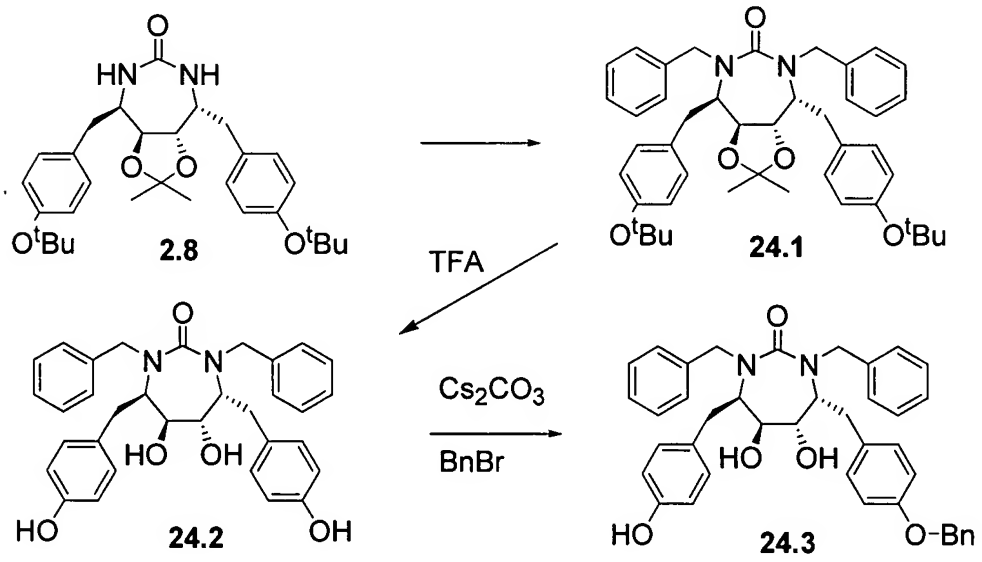
Phenol 23.6: A solution of **23.5** (0.28 g, 0.593 mmol) in ethyl acetate (5 mL) and isopropyl alcohol (5 mL) was treated with 10% Pd/C (0.05g) and stirred under a hydrogen atmosphere (balloon) for 16 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to yield **23.6** (0.22 g, 97%) as a white solid.

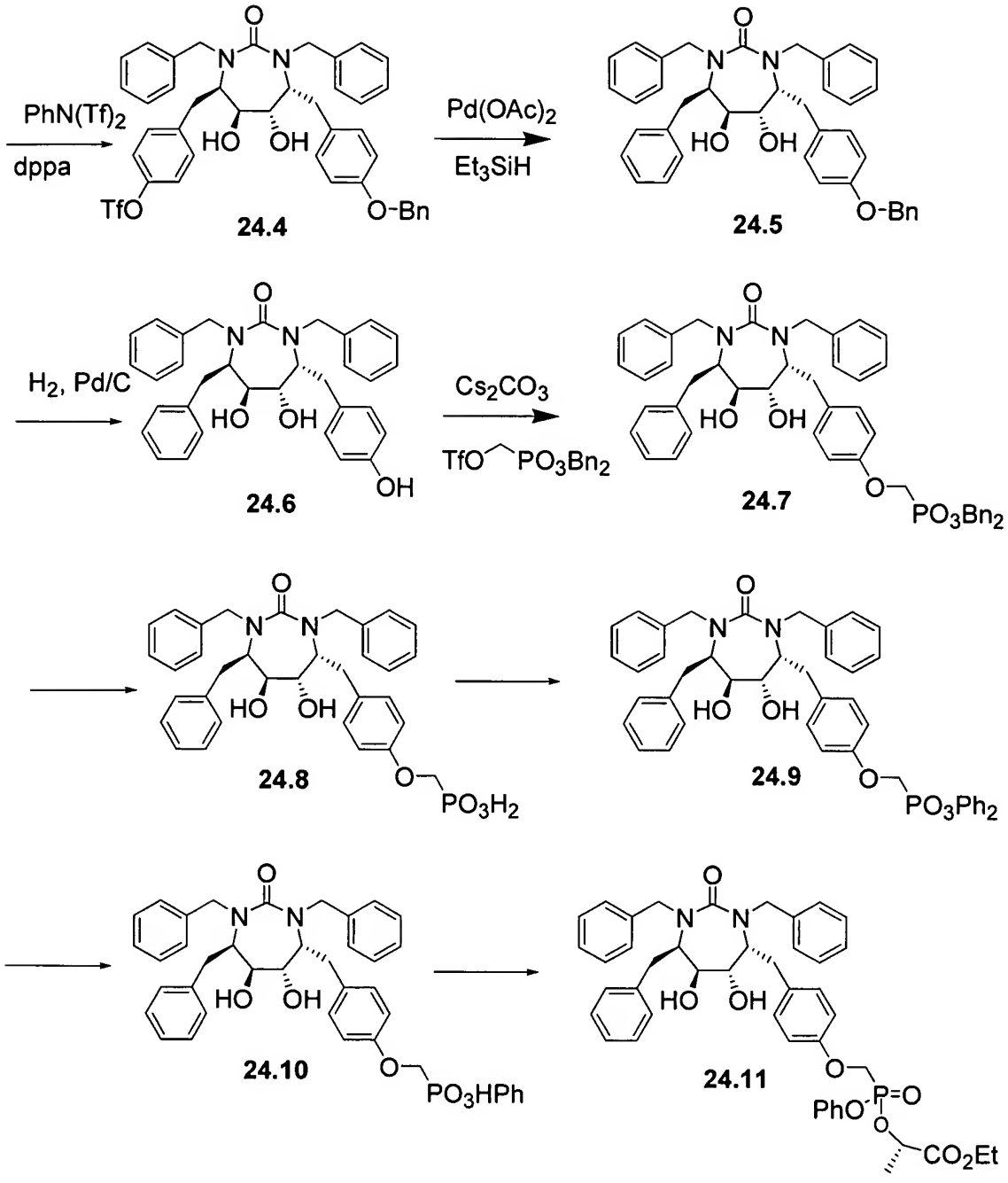
Dibenzyl phosphonate 23.7: To a solution of compound **23.6** (0.215 g, 0.563 mmol) in THF (10 mL) was added dibenzyl triflate **3.11** (0.315 g, 0.74 mmol) and cesium carbonate (0.325g, 1 mmol). The mixture was stirred at room temperature for 2 h, then diluted with ethyl acetate and washed with water. The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford compound **23.7** (0.31 g, 84%).

Diphenyl ester 23.8: A solution of compound **23.7** (0.3 g, 0.457 mmol) and benzyl bromide (0.165 mL, 1.39 mmol) in THF (10 mL) was treated with potassium *tert*-butoxide (1M/THF, 1.2 mL) for 0.5 h. The mixture was diluted with ethyl acetate and washed with HCl (0.2N). The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was dissolved in ethyl acetate and treated with 10% Pd/C (0.05 g) under hydrogen atmosphere (balloon) for 16 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was treated with TFA (1 mL) in methanol (5 mL) for 1 h, and then concentrated under reduced pressure. The residue was dissolved in pyridine (1 mL) and mixed with phenol (0.45 g, 4.8 mmol) and 1,3-dicyclohexylcarbodiimide (0.38 g, 1.85 mmol). The mixture was stirred at 70°C for 2 h, and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate and HCl (0.2N). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was purified by chromatography on silica gel to afford compound **23.8** (0.085 g, 24%).

Mono amidate 23.9: To a solution of **23.8** (0.085g, 0.11 mmol) in acetonitrile (1 mL) was added sodium hydroxide (1N, 0.25 mL) at 0°C. After stirred at 0°C for 1 h, the mixture was acidified with Dowex resin to pH = 3, and filtered. The filtrate was concentrated under reduced pressure. The residue was dissolved in pyridine (0.5 mL) and mixed with L-alanine ethyl ester hydrochloride (0.062 g, 0.4 mmol) and 1,3-dicyclohexyl-carbodiimide (0.125 g, 0.6 mmol). The mixture was stirred at 60°C for 0.5 h, and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate and HCl (0.2N). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was purified by HPLC (C-18, 65% acetonitrile / water) to afford compound **23.9** (0.02 g, 23%). ^{1}H NMR (CDCl3): δ 1.2 (m, 3H), 1.4 (m, 3H), 1.8 (brs, 2H), 2.8-3.1 (m, 6H), 3.5-3.7 (m, 4H), 3.78 (m, 1H), 4.0-4.18 (m, 2H), 4.2-4.4 (m, 3H), 4.9 (m, 2H), 6.8-7.4 (m, 24H). 31P NMR (CDCl3): d 20.9, 19.8. MS: 792 (M+1).

Scheme 24





Di-tert butyl ether 24.1: To a solution of compound **2.8** (0.51 g, 1 mmol) and benzyl bromide (0.43g, 2.5 mmol) in THF (6 mL) was added potassium *tert*-butoxide (1M/THF, 2.5 mL). The mixture was stirred at room temperature for 0.5 h, then diluted with ethyl acetate and washed with water. The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford compound **24.1** (0.62 g, 90%).

Diol 24.2: To a solution of compound **24.1** (0.62 g, 0.9 mmol) in methylene chloride (4 mL) was added TFA (1 mL) and water (0.1 mL). The mixture was stirred for 2 h, and then concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford compound **24.2** (0.443g, 92%).

Benzyl ether 24.3: Compound **24.3** was prepared in 46% yield according to the procedure described in Scheme 23 for the preparation of **23.3**.

Triflate 24.4: Compound **24.4** was prepared in 95% yield according to the procedure described in Scheme 23 for the preparation of **23.4**.

Benzyl ether 24.5: Compound **24.5** was prepared in 93% yield according to the procedure described in Scheme 23 for the preparation of **23.5**.

Phenol 24.6: Compound **24.6** was prepared in 96% yield according to the procedure described in Scheme 23 for the preparation of **23.6** from **23.5**.

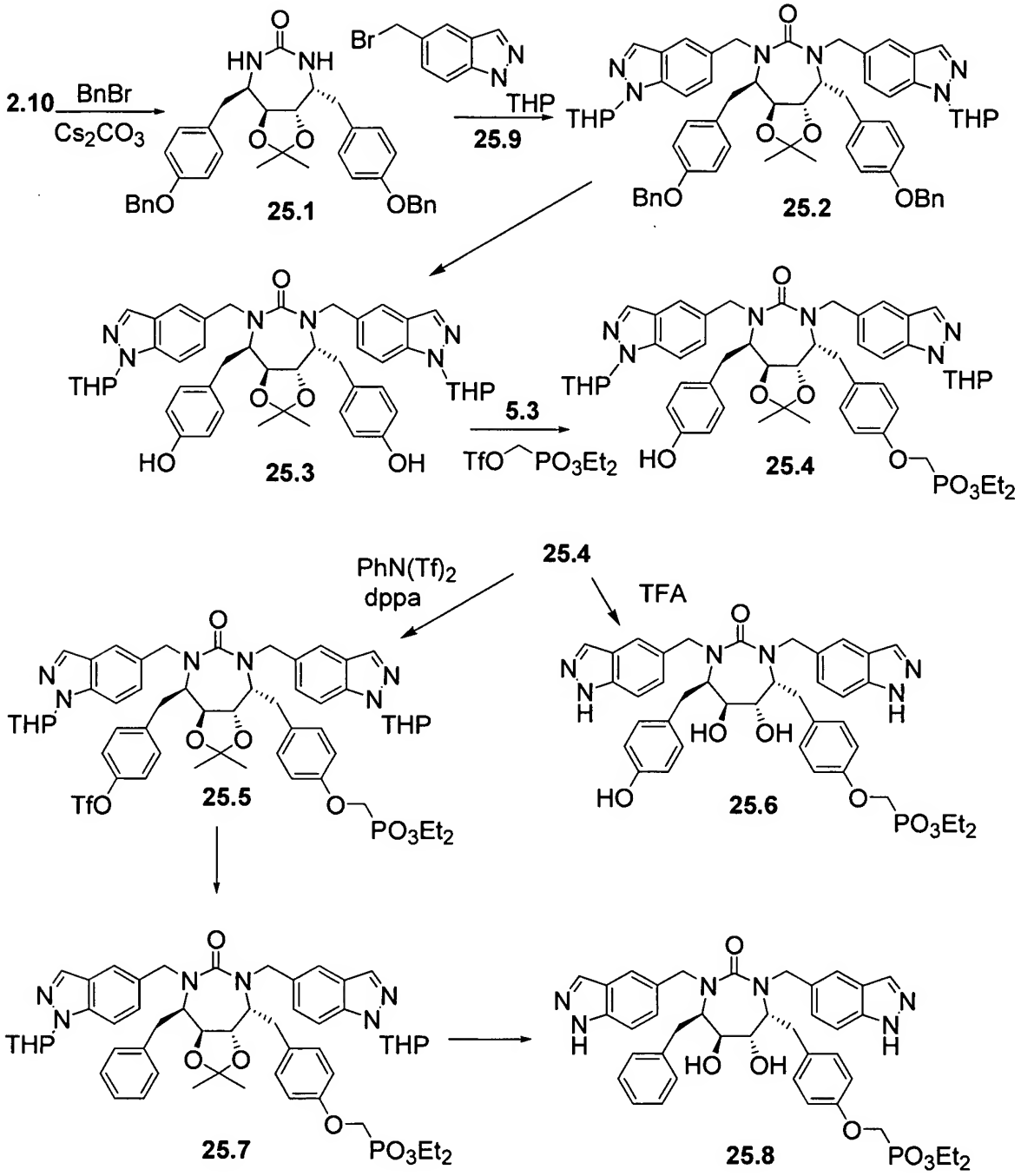
Dibenzyl phosphonate 24.7: Compound **24.7** was prepared in 82% yield according to the procedure described in Scheme 23 for the preparation of **23.7**.

Diacid 24.8: A solution of **24.7** (0.16 g, 0.207 mmol) in ethyl acetate (4 mL) and isopropyl alcohol (4 mL) was treated with 10% Pd/C (0.05g) and stirred under a hydrogen atmosphere (balloon) for 4 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to yield **24.8** (0.125 g, 98%) as a white solid.

Diphenyl ester 24.9: To a solution of compound **24.8** (0.12 g, 0.195 mmol) in pyridine (1 mL) was added phenol (0.19 g, 2 mmol) and 1,3-dicyclohexylcarbodiimide (0.206 g, 1 mmol). The mixture was stirred at 70°C for 2 h, and then concentrated under reduced pressure. The residue was partitioned between ethyl acetate and HCl (0.2N). The organic phase was dried over magnesium sulfate, filtered and concentrated. The residue was purified by chromatography on silica gel to afford compound **24.9** (0.038 g, 25%).

Mono lactate 24.11: Compound **24.9** was converted, via compound **24.10**, into compound **24.11** in 36% yield according to the procedure described in Scheme 23 for the preparation of **23.9** except utilizing the ethyl lactate ester in place of L-alanine ethyl ester . ^{1}H NMR (CDCl3): δ 1.05 (t, J = 8 Hz, 1.5H), 1.1 (t, J = 8 Hz, 1.5H), 1.45 (d, J = 8 Hz, 1.5H), 1.55 (d, J = 8 Hz, 1.5H), 2.6 (brs, 2H), 2.9-3.1 (m, 6H), 3.5-3.65 (m, 4H), 4.15-4.25 (m, 2H), 4.4-4.62 (m, 2H), 4.9 (m, 2H), 5.2 (m, 1H), 6.9-7.4 (m, 24H). 31P NMR (CDCl3): d 17.6, 15.5. MS: 793 (M+1).

Scheme 25



Dibenzyl ether 25.1: The protection reaction of compound **2.10** with benzyl bromide was carried out in the same manner as described in Scheme 23 to afford compound **25.1**.

Bis indazole 25.2: The alkylation of compound **25.1** with bromide **25.9** was carried out in the same manner as described in Scheme 23 to afford compound **25.2** in 96% yield.

Diol 25.3: A solution of **25.2** (0.18 g, 0.178 mmol) in ethyl acetate (5 mL)) and isopropyl alcohol (5 mL) was treated with 20% Pd(OH)2/C (0.09g) and stirred under a hydrogen atmosphere (balloon) for 24 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to afford **25.3** in quantitative yield.

Diethyl phosphonate 25.4: To a solution of compound **25.3** (0.124 g, 0.15 mmol) in acetonitrile (8 mL) and DMF (1 mL) was added potassium tert-butoxide (0.15 mL, 1M/THF). The mixture was stirred for 10 min. to form a clear solution. Diethyl triflate **5.3** (0.045 g, 0.15 mmol) was added to the reaction mixture. After stirred for 0.5 h, the reaction mixture was diluted with ethyl acetate and washed with HCl (0.1N). The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford compound **25.4** (0.039 g, 55% (based on recovered starting material: 0.064 g, 52%).

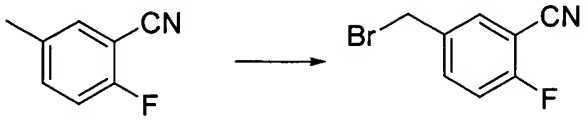
Bisindazole 25.6: A mixture of compound **25.4** (0.027 g), ethanol (1.5 mL), TFA (0.6 mL) and water (0.5 mL) was stirred at 60°C for 18 h. The mixture was concentrated under reduced pressure, and the residue was purified by HPLC to afford compound **25.6** as a TFA salt (0.014 g, 51%). ^{1}H NMR (CD3OD): δ 1.4 (t, J = 8 Hz, 6H), 2.9 (M, 4H), 3.2 (m, 2H), 3.58 (brs, 2H), 3.65 (m, 2H), 4.25 (m, 4H), 4.42 (d, J = 10 Hz, 2H), 4.85 (m, 2H), 6.75 (d, J = 9 Hz, 2H), 6.9 (m, 4H), 7.0 (d, J = 9 Hz, 2H), 7.4-7.6 (m, 6H), 8.1 (brs, 2H). 31P NMR (CD3OD): δ 20.8. MS: 769 (M+1).

Diethyl phosphonate 25.7: Compound **25.4** was converted into compound **25.7** in 76% yield according to the procedures described in Scheme 23 for the conversion of **23.3** into **23.5**.

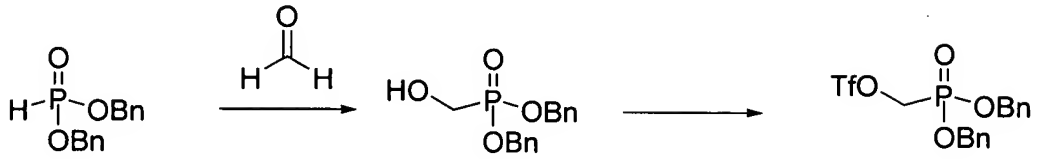
Bis indazole 25.8: Compound **25.7** (0.029 g) was treated in the same manner as compound **25.4** in the preparation of **25.6** to afford compound **25.8** as a TFA salt (0.0175 g, 59%). ^{1}H NMR (CD3OD): δ 1.4 (t, J = 8 Hz, 6H), 3.0 (M, 4H), 3.15 (d, J = 14 Hz, 1H), 3.25 (d, J = 14 Hz, 1H), 3.58 (brs, 2H), 3.65 (m, 2H), 4.25 (m, 4H), 4.42 (d, J = 10 Hz, 2H), 4.85 (m, 2H), 6.9 (d, J = 9 Hz, 2H), 7.0 (d, J = 9 Hz, 2H), 7.1 (d, J = 7 Hz, 2H), 7.2-7.6 (m, 9H), 8.1 (brs, 2H). ^{31}P NMR (CD3OD): δ 20.8. MS: 753 (M+1).

Preparation of Alkylating and Phosphonate Reagents

Scheme 50

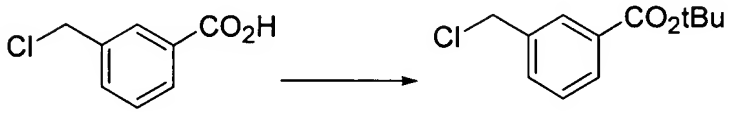


50.1 **3.9**

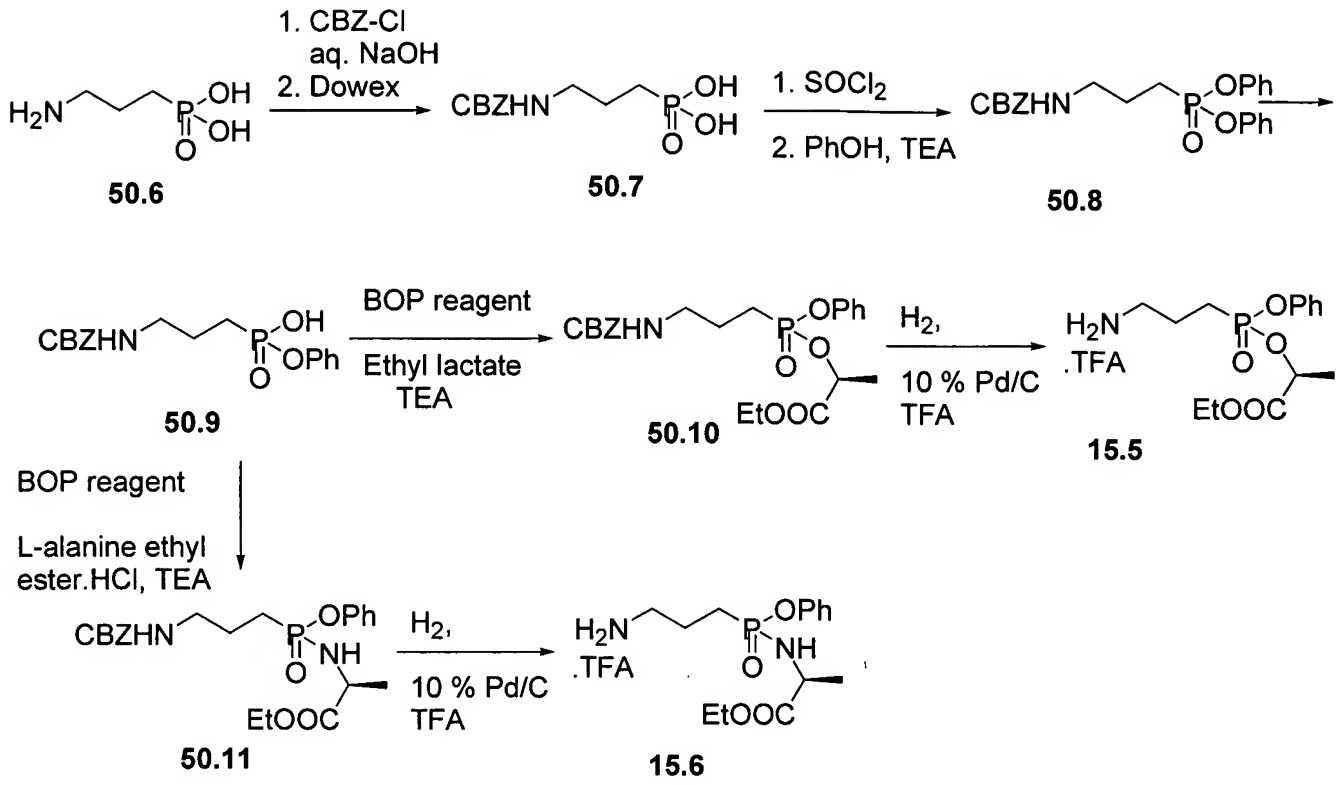


50.2 **50.3** **3.11**

50.4 **6.9**



50.5 **14.5**



50.6 **50.7** **50.8** **50.9** **50.10** **15.5** **50.11** **15.6**

3-cyano-4-fluoro-benzylbromide 3.9: The commercially available 2—fluoro-4-methylbenzonitrile **50.1** (10 g, 74 mmol) was dissolved in carbon tetrachloride (50 mL) and then treated with NBS (16 g, 90 mmol) followed by AIBN (0.6 g, 3.7 mmol). The mixture was stirred at 85°C for 30 min and then allowed to cool to room temperature. The mixture was filtered and the filtrate concentrated under reduced pressure. The residue was purified by silica gel eluting with 5-20% ethyl acetate in hexanes to give **3.9** (8.8 g, 56%).

4-benzyloxy benzyl chloride 3.10 is purchased from Aldrich.

Dibenzyl triflate 3.11: To a solution of dibenzyl phosphite **50.2** (100 g, 381 mmol) and formaldehyde (37% in water, 65 mL, 860 mmol) in THF (200 mL) was added TEA (5 mL, 36 mmol). The resulted mixture was stirred for 1 h, and then concentrated under reduced pressure. The residue was dissolved in methylene chloride and hexane (1:1, 300 mL), dried over sodium sulfate, filtered through a pad of silica gel (600 g) and eluted with ethyl acetate and hexane (1:1). The filtrate was concentrated under reduced pressure. The residue **50.3** (95 g) was dissolved in methylene chloride (800 mL), cooled to –78°C and then charged with pyridine (53 mL, 650 mmol). To this cooled solution was slowly added trifluoromethanesulfonic anhydride (120 g, 423 mmol). The resulted reaction mixture was stirred and gradually warmed up to –15°C over

1.5 h period of time. The reaction mixture was cooled down to about – 50°C, diluted with hexane-ethyl acetate (2:1, 500 mL) and quenched with aqueous phosphoric acid (1M, 100 mL) at –10°C to 0°C. The mixture diluted with hexane-ethyl acetate (2:1, 1000 mL). The organic phase was washed with water, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to afford dibenzyl triflate **3.11** (66 g, 41%) as a colorless oil.

Diethyl triflate 5.3 is prepared as described in *Tetrahedron Lett.* 1986, 27, p1477-1480.

3-Benzyloxybenzylbromide 6.9: To a solution of triphenyl phosphine (15.7 g, 60 mmol) in THF (150 mL) was added a solution of carbon tetrabromide (20 g, 60 mmol) in THF (50 mL). A precipitation was formed and stirred for 10 min. A solution of 3-benzyloxybenzyl alcohol **50.4** (10 g, 46.7 mmol) was added. After stirred for 1.5 h, the reaction mixture was filtered and concentrated under reduced pressure. The majority of triphenyl phosphine oxide was removed by precipitation from ethyl acetate-hexane. The crude product was purified by chromatography on silica gel and precipitation from hexane to give the desired product 3-Benzyloxybenzylbromide **6.9** (10 g, 77%) as a white solid.

t-Butyl-3-chloromethyl benzoate 14.5: A benzene solution (15 ml) of 3-chloromethylbenzoic acid **50.5** (1 g, 5.8 mmol) was heated at reflux, followed by the slow addition of N,N-dimethylforamide-di-t-butylacetal (5 m). The resulting solution was refluxed for 4 h, concentrated under reduced pressure and purified by silica gel column to afford **14.5** (0.8 g, 60 %).

Aminopropyl-diethylphosphonate 14.6 is purchased from Acros.

Aminoethyl-diethylphosphonate oxalate 14.7 is purchased from Acros.

Aminopropyl-phenol-ethyl lactate phosphonate 15.5

N-CBZ-aminopropyl diphenylphosphonate 50.8: An aqueous sodium hydroxide solution (50 mL of 1 N solution, 50 mmol) of 3-aminopropyl phosphonic acid **50.6** (3 g, 1.5 mmol) was reacted with CBZ-Cl (4.1 g, 24 mmol) at room temperature overnight. The reaction mixture was washed with methylene chloride, acidified with Dowex 50wx8-200. The resin was filtered off. The filtrate was concentrated to dryness. The crude N-CBZ-aminopropyl phosphonic acid **50.7** (5.8 mmol) was suspended in CH_3CN (40 mL), and reacted with thionyl chloride (5.2 g, 44 mmol) at reflux for 4 hr, concentrated, and azeotroped with CH_3CN twice. The reaction mixture was redissolved in methylene chloride (20 mL), followed by the addition of

phenol (3.2 g, 23 mmol), was cooled to 0°C. To this 0°C cold solution was added TEA (2.3 g, 23 mmol), and stirred at room temperature overnight. The reaction mixture was concentrated and purified on silica gel column chromatograph to afford **50.8** (1.5 g, 62 %).

Monophenol derivative 50.9: A CH_3CN solution (5 mL) of **50.8** (0.8 g, 1.88 mmol) was cooled to 0°C , and treated with 1N NaOH aqueous solution (4 mL, 4 mmol) for 2 h. The reaction was diluted with water, extracted with ethyl acetate, acidified with Dowex 50wx8-200. The aqueous solution was concentrated to dryness to afford **50.9** (0.56 g, 86%).

Monolactate derivative 50.10: A DMF solution (1 mL) of crude **50.9** (0.17 g, 0.48 mmol), BOP reagent (0.43 g, 0.97 mmol), ethyl lactate (0.12 g, 1 mmol), and DIPEA (0.31 g, 2.4 mmol) was reacted for 4 hr at room temperature. The reaction mixture was partitioned between methylene chloride and 5 % citric acid aqueous solution. The organic solution was separated, concentrated, and purified on preparative TLC to give **50.10** (0.14 g, 66%).

3-Aminopropyl lactate phosphonate 15.5: An ethyl acetate/ethanol solution (10 mL/2 mL) of **50.10** (0.14 g, 0.31 mmol) was hydrogenated at 1 atm in the presence of 10% Pd/C (40 mg) for 3 hr. The catalyst was filtered off. The filtrate was concentrated to dryness to afford **15.5** (0.14 g, quantitative). NMR ($CDCl_3$): δ 8.0-8.2 (b, 3H), 7.1-7.4 (m, 5H), 4.9-5.0 (m, 1H), 4.15-4.3 (m, 2H), 3.1-3.35 (m, 2H), 2.1-2.4 (m, 4H), 1.4 (d, 3H), 1.3 (t, 3H).

Aminopropyl-phenol-ethyl alanine phosphonate 15.6: Compound **15.6** (80 mg) was prepared from the reaction of **50.9** (160 mg, 0.45 mmol) and L-alanine ethyl ester hydrochloride salt (0.11g, 0.68 mmol) in the presence of DIPEA and BOP reagent to give **50.11**, followed by the hydrogenation in the presence of 10% Pd/C and TFA to yield **15.6**. NMR ($CDCl_3$ + ~10 % CD_3OD): δ 8.0-8.2 (b), 7.25-7.35 (t, 2H), 7.1-7.2 (m, 3H), 4.0-4.15 (m, 2H), 3.8-4.0 (m, 1H), 3.0-3.1 (m, 2H), 1.15-1.25 (m, 6H). P NMR ($CDCl_3$ + ~10 % CD_3OD): 32.1 & 32.4 ppm.

Aminopropyl dibenzyl phosphonate 15.7 :

N-BOC-3-aminopropyl phosphonic acid 50.13: A THF-1N aqueous solution (16 mL-16 mL) of 3-aminopropyl phosphonic acid **50.12** (1 g, 7.2 mmol) was reacted with $(BOC)_2O$ (1.7 g, 7.9 mmol) overnight at room temperature. The reaction mixture was concentrated, and partitioned between methylene chloride and water. The aqueous solution was acidified with Dowex 50wx8-200. The resin was filtered off. The filtrate was concentrated to give **50.13** (2.2 g, 92 %).

N-BOC-3-aminopropyl dibenzyl phosphonate 50.14: A CH_3CN solution (10 mL) of **50.13** (0.15 g, 0.63 mmol), cesium carbonate (0.61 g, 1.88 mmol), and benzyl bromide (0.24 g, 1.57 mmol) was heated at reflux overnight. The reaction mixture was cooled to room temperature, and diluted with methylene chloride. The white solid was filtered off, washed thoroughly with methylene chloride. The organic phase was concentrated, and purified on preparative TLC to give **50.14** (0.18 g, 70%). MS: 442 (M + Na).

Aminopropyl dibenzyl phosphonate 15.7: A methylene chloride solution (1.6 mL) of **50.14** (0.18 g) was treated with TFA (0.4 mL) for 1 hr. The reaction mixture was concentrated to dryness, and azeotroped with CH_3CN twice to afford **15.7** (0.2 g, as TFA salt). NMR ($CDCl_3$): δ 8.6 (b, 2H), 7.9 (b, 2H), 7.2-7.4 (m, 10H), 4.71-5.0 (2 abq, 4H), 3.0 (b, 2H), 1.8-2 (m, 4H). 31P NMR ($CDCl_3$): 32.0 ppm. F NMR ($CDCl_3$): -76.5 ppm.

Aminomethyl diethylphosphonate 22.8 is purchased from Acros.

Bromomethyl, tetrahydropyran indazole 25.9 is prepared according to *J. Org. Chem.* 1997, 62, p5627.

Activity of the CCPPI Compounds

The enzyme inhibitory potency (Ki), antiviral activity (EC50), and cytotoxicity (CC50) of the tested compounds were measured and demonstrated.

Biological assays used for the characterization of PI prodrugs

HIV-1 Protease Enzyme Assay (*Ki*)

The assay is based on the fluorimetric detection of synthetic hexapeptide substrate cleavage by HIV-1 protease in a defined reaction buffer as initially described by M.V.Toth and G.R.Marshall, *Int. J. Peptide Protein Res.* 36, 544 (1990).

Substrate: (2-aminobenzoyl)Thr-Ile-Nle-(p-nitro)Phe-Gln-Arg
Substrate supplied by Bachem California, Inc. (Torrance, CA; Cat. no. H-2992)

Enzyme: recombinant HIV-1 protease expressed in *E.Coli*
Enzyme supplied by Bachem California, Inc. (Torrance, CA; Cat. no. H-9040)

Reaction buffer: 100 mM ammonium acetate, pH 5.3
1 M sodium chloride

1 mM ethylendiaminetetraacetic acid

1 mM dithiothreitol

10% dimethylsulfoxide

Assay protocol for the determination of inhibition constant Ki:

1. Prepare series of solutions containing identical amount of the enzyme (1 to 2.5 nM) and a tested inhibitor at different concentrations in the reaction buffer.
2. Transfer the solutions (190 uL each) into a white 96-well plate.
3. Preincubate for 15 min at 37°C.
4. Solubilize the substrate in 100% dimethylsulfoxide at a concentration of 800 μM. Start the reaction by adding 10 μL of 800 μM substrate into each well (final substrate concentration of 40 μM).
5. Measure the real-time reaction kinetics at 37°C by using Gemini 96-well plate fluorimeter (Molecular Devices, Sunnyvale, CA) at λ(Ex) = 330 nm and λ(Em) = 420 nm.
6. Determine initial velocities of the reactions with different inhibitor concentrations and calculate Ki (in picomolar concentration units) value by using EnzFitter program (Biosoft, Cambridge, U.K.) according to an algorithm for tight-binding competitive inhibition described by Ermolieff J., Lin X., and Tang J., *Biochemistry* 36, 12364 (1997).

Anti-HIV-1 Cell Culture Assay (EC_{50})

The assay is based on quantification of the HIV-1-associated cytopathic effect by a colorimetric detection of the viability of virus-infected cells in the presence or absence of tested inhibitors. The HIV-1-induced cell death is determined using a metabolic substrate 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) which is converted only by intact cells into a product with specific absorption characteristics as described by Weislow OS, Kiser R, Fine DL, Bader J, Shoemaker RH and Boyd MR, *J. Natl. Cancer Inst.* 81, 577 (1989).

Assay protocol for determination of EC_{50}:

1. Maintain MT2 cells in RPMI-1640 medium supplemented with 5% fetal bovine serum and antibiotics.

2. Infect the cells with the wild-type HIV-1 strain IIIB (Advanced Biotechnologies, Columbia, MD) for 3 hours at 37°C using the virus inoculum corresponding to a multiplicity of infection equal to 0.01.
3. Prepare a set of solutions containing various concentrations of the tested inhibitor by making 5-fold serial dilutions in 96-well plate (100 μL/well). Distribute the infected cells into the 96-well plate (20,000 cells in 100 μL/well). Include samples with untreated infected and untreated mock-infected control cells.
4. Incubate the cells for 5 days at 37°C.
5. Prepare XTT solution (6 mL per assay plate) at a concentration of 2mg/mL in a phosphate-buffered saline pH 7.4. Heat the solution in water-bath for 5 min at 55°C. Add 50 μL of N-methylphenazonium methasulfate (5 μg/mL) per 6 mL of XTT solution.
6. Remove 100 μL media from each well on the assay plate.
7. Add 100 μL of the XTT substrate solution per well and incubate at 37°C for 45 to 60 min in a CO_2 incubator.
8. Add 20 μL of 2% Triton X-100 per well to inactivate the virus.
9. Read the absorbance at 450 nm with subtracting off the background absorbance at 650 nm.
10. Plot the percentage absorbance relative to untreated control and estimate the EC_{50} value as drug concentration resulting in a 50% protection of the infected cells.

Cytotoxicity Cell Culture Assay (CC_{50})

The assay is based on the evaluation of cytotoxic effect of tested compounds using a metabolic substrate 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) as described by Weislow OS, Kiser R, Fine DL, Bader J, Shoemaker RH and Boyd MR, *J. Natl. Cancer Inst.* 81, 577 (1989).

Assay protocol for determination of CC_{50}:

1. Maintain MT-2 cells in RPMI-1640 medium supplemented with 5% fetal bovine serum and antibiotics.
2. Prepare a set of solutions containing various concentrations of the tested inhibitor by making 5-fold serial dilutions in 96-well plate (100 μL /well). Distribute cells into the 96-well plate (20,000 cells in 100 μL/well). Include samples with untreated cells as a control.

3. Incubate the cells for 5 days at 37°C.
4. Prepare XTT solution (6 mL per assay plate) in dark at a concentration of 2mg/mL in a phosphate-buffered saline pH 7.4. Heat the solution in a water-bath at 55°C for 5 min. Add 50 μL of N-methylphenazonium methasulfate (5 μg/mL) per 6 mL of XTT solution.
5. Remove 100 μL media from each well on the assay plate and add 100 μL of the XTT substrate solution per well. Incubate at 37°C for 45 to 60 min in a CO_2 incubator.
6. Add 20 μL of 2% Triton X-100 per well to stop the metabolic conversion of XTT.
7. Read the absorbance at 450 nm with subtracting off the background at 650 nm.
8. Plot the percentage absorbance relative to untreated control and estimate the CC50 value as drug concentration resulting in a 50% inhibition of the cell growth. Consider the absorbance being directly proportional to the cell growth.

Resistance Evaluation (I50V and I84V/L90M fold change)

The assay is based on the determination of a difference in the susceptibility to a particular HIV protease inhibitor between the wild-type HIV-1 strain and a mutant HIV-1 strain containing specific drug resistance-associated mutation(s) in the viral protease gene. The absolute susceptibility of each virus (EC_{50}) to a particular tested compound is measured by using the XTT-based cytopathic assay as described above. The degree of resistance to a tested compound is calculated as fold difference in EC_{50} between the wild type and a specific mutant virus. This represents a standard approach for HIV drug resistance evaluation as documented in various publications (e.g. Maguire *et al.*, *Antimicrob. Agents Chemother.* 46: 731, 2002; Gong *et al.*, *Antimicrob. Agents Chemother.* 44: 2319, 2000; Vandamme and De Clercq, in Antiviral Therapy (Ed. E. De Clercq), pp. 243, ASM Press, Washington, DC, 2001).

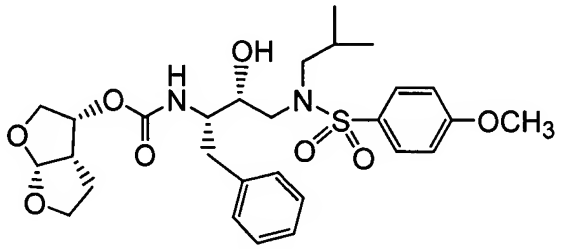
HIV-1 strains used for the resistance evaluation

Two strains of mutant viruses containing I50V mutation in the protease gene have been used in the resistance assays: one with M46I/I47V/I50V mutations (designated I50V #1) and the other with L10I/M46I/I50V (designated I50V #2) mutations in the viral protease gene. A third virus with I84V/L90M mutations was also employed in the resistance assays. Mutants I50V #1 and I84V/L90M were constructed by a homologous recombination between three overlapping DNA fragments: 1. linearized plasmid containing wild-type HIV-1 proviral DNA (strain

HXB2D) with the protease and reverse transcriptase genes deleted, 2. DNA fragment generated by PCR amplification containing reverse transcriptase gene from HXB2D strain (wild-type), 3. DNA fragment of mutated viral protease gene that has been generated by PCR amplification. An approach similar to that described by Shi and Mellors in *Antimicrob. Agents Chemother.* 41: 2781-85, 1997 was used for the construction of mutant viruses from the generated DNA fragments. Mixture of DNA fragments was delivered into Sup-T1 cells by using a standard electroporation technique. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics until the recombinant virus emerged (usually 10 to 15 days following the electroporation). Cell culture supernatant containing the recombinant virus was harvested and stored in aliquots. After verification of protease gene sequence and determination of the infectious virus titer, the viral stock was used for drug resistance studies. Mutant I50V #2 is an amprenavir-resistant HIV-1 strain selected *in vitro* from the wild-type IIIB strain in the presence of increasing concentration of amprenavir over a period of > 9 months using an approach similar to that described by Partaledis *et al., J. Virol.* 69: 5228-5235, 1995. Virus capable of growing in the presence of 5 μM amprenavir was harvested from the supernatant of infected cells and used for resistance assays following the titration and protease gene sequencing.

Example 37: Activity of the Tested Compounds

The enzyme inhibitory potency (Ki), antiviral activity (EC50), and cytotoxicity (CC50) of the tested compounds are summarized in Table 1.



94-003

Table 1: Enzyme inhibition activity (Ki), antiviral cell culture activity (EC50), and cytotoxicity (CC50) of the tested compounds

Substitution of (P1)phenyl	Compound	Phosphonate substitution	HIV-1 protease inhibition Ki [pM]	Anti-HIV-1 Cell Culture Activity EC50 [nM]	Cytotoxicity CC50 [μM]
none	Amprenavir	none	45.6 ± 18.2	16 ± 2.2	
none	94-003	none	1.46 ± 0.58	1.4 ± 0.3	
phosphonyl	27	diacid	11.8 ± 6.0	> 100,000	> 100
	28	diethyl	1.2 ± 0.8	5.0 ± 2.8	70
phosphonyl methoxy	11	diacid	2.1 ± 0.2	4,800 ± 1,800	> 100
	13	diethyl	2.6 ± 1.5	3.0 ± 0	50
	14	dibenzyl	12.7 ± 1.9	2.3 ± 0.4	35
	16c	bis(Ala-ethylester)	15.4 ± 0.85	105 ± 43	60
	16d	bis(Ala-butylester)	18.75 ± 3.04	6.0 ± 1.4	
	16e	bis(ABA-ethylester)	8.8 ± 1.7	12.5 ± 3.5	
	16f	bis(ABA-butylester)	3.5 ± 1.4	4.8 ± 1.8	
	16a	bis(Gly-ethylester)	29 ± 8.2	330 ± 230	
	16b	bis(Gly-butylester)	4.9 ± 1.8	17.5 ± 10.5	
	16g	bis(Leu-ethylester)	29 ± 9	6.8 ± 0.4	
	16h	bis(Leu-butylester)	31.7 ± 19.3	120 ± 42	
	16i	bis(Phe-ethylester)		17 ± 12	
	16j	bis(Phe-butylester)		35 ± 7	
	15	bis(POC)	36	825 ± 106	
	11	Monoethyl, monoacid	0.45 ± 0.15	700 ± 0	

Cross-Resistance Profile Assay

The assay is based on the determination of a difference in the susceptibility to a particular HIV protease inhibitor between the wild-type HIV-1 strain and a recombinant HIV-1 strain expressing specific drug resistance-associated mutation(s) in the viral protease gene. The

absolute susceptibility of each virus to a particular tested compound is measured by using the XTT-based cytopathic assay as described in Example B. The degree of resistance to a tested compound is calculated as fold difference in EC50 between the wild type and a specific mutant virus.

Recombinant HIV-1 strains with resistance mutations in the protease gene

One mutant virus (82T/84V) was obtained from NIH AIDS Research and Reference Reagent Program (Rockville, MD). Majority of the mutant HIV-1 strains were constructed by a homologous recombination between three overlapping DNA fragments: 1. linearized plasmid containing wild-type HIV-1 proviral DNA (strain HXB2D) with the protease and reverse transcriptase genes deleted, 2. DNA fragment generated by PCR amplification containing reverse transcriptase gene from HXB2D strain (wild-type), 3. DNA fragment generated by RT-PCR amplification from patients plasma samples containing viral protease gene with specific mutations selected during antiretroviral therapy with various protease inhibitors. Additional mutant HIV-1 strains were constructed by a modified procedure relying on a homologous recombination of only two overlapping DNA fragments: 1. linearized plasmid containing wild-type HIV-1 proviral DNA (strain HXB2D) with only the protease gene deleted, and 2. DNA fragment generated by RT-PCR amplification from patients plasma samples containing viral protease gene with specific mutations. In both cases, mixture of DNA fragments was delivered into Sup-T1 cells by using a standard electroporation technique. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics until the recombinant virus emerged (usually 10 to 15 days following the electroporation). Cell culture supernatant containing the recombinant virus was harvested and stored in aliquots. After determination of the virus titer the virus stock was used for drug resistance studies.

Example 39: Cross-Resistance Profile of the Tested Compounds

Cross-resistance profile of currently used HIV-1 protease inhibitors was compared with that of the newly invented compounds (Table 2).

Table 2: Cross-resistance profile of HIV-1 protease inhibitors

		Fold Change in EC_{50} Relative to WT HIV-1											
Compound	EC 50 [nM] WT HIV-1	8K[a] 46I 90M	46I 84A	10I 48V 54V 82A	46I 47V 50V	10R 46I 82T 84V	30N 50S 82I 88D	54V 71V 82S	10F 46I 71V 82T 90M	10I 48V 71V 82A 90M	48V 54V 71V 82S	10I 84V 71V 73S 90M	Total No. of Resis-tant Viruses[b]
Amprenavir	20	1.25	**14**	2	**38**	4	0.8	4	**13**	2.5	2	**10**	4
Nelfinavir	14	**13**	**11**	**11.5**	2	3	**43**	**12**	**33**	**27**	**12**	**65**	9
Indinavir	15	4	**10**	**15**	nd	**7**	1	**10**	**13**	**28**	**23**	**43**	8
Ritonavir	15	**34**	**18**	**20**	**13**	**47**	2	**20**	**32**	**22**	**>50**	**42**	10
Saquinavir	4	1	2.5	**11**	1	2.5	1	3	2.5	**12**	**45**	**40**	4
Lopinavir	8	nd	**9**	nd	**19**	**11**	nd	nd	**7.5**	4.5	**60**	**11**	6
Tipranavir	80	nd	1	0.4	0.5	**5**	0.5	3.5	3	0.3	2	**nd**	1
94-003	0.5	nd	**8**	0.5	**29**	nd	0.4	3.5	nd	nd	nd	**8**	3
GS 16503	16	1.2	1	0.4	3.3	1	0.6	0.9	1	0.4	0.5	2	0
GS 16571	22	1.8	1	0.3	0.8	0.6	0.7	0.6	0.8	0.2	0.2	0.9	0
GS 16587	15	1.5	1	0.5	2	1	1	0.9	1	0.4	0.4	1	0

[a] Resistance-associated mutations present in the viral protease. The highlighted changes primary resistance mutations.

[b] Resistance is considered as a 5-fold and higher change in the EC50 value of the mutant virus relative to the wild-type virus.

Plasma and PBMC Exposure Following Intravenous and Oral Administration of Prodrug to Beagle Dogs

The pharmacokinetics of a phosphonate prodrug GS77366 (P1-monoLac-iPr), its active metabolite (metabolite X, or GS77568), and GS8373 were studied in dogs following intravenous and oral administration of the prodrug.

Dose Administration and Sample Collection

The in-life phase of this study was conducted in accordance with the USDA Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and followed the standards for animal husbandry and care found in the Guide for the Care and Use of Laboratory Animals, 7th Edition, Revised 1996. All animal housing and study procedures involving live animals were carried out at a facility which had been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care - International (AAALAC).

Each animal in a group of 4 female beagle dogs was given a bolus dose of GS77366 (P1-monoLac-iPr) intravenously at 1 mg/kg in a formulation containing 40% PEG 300, 20% propylene glycol and 40% of 5% dextrose. Another group of 4 female beagle dogs was dosed with GS77366 via oral gavage at 20 mg/kg in a formulation containing 60% Vitamin-E TPGS, 30% PEG 400 and 10% propylene glycol.

Blood samples were collected pre-dose, and at 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr and 24 hr post-dose. Plasma (0.5 to 1 mL) was prepared from each sample and kept at -70°C until analysis. Blood samples (8 mL) were also collected from each dog at 2, 8 and 24 hr post dose in Becton-Dickinson CPT vacutainer tubes. PBMCs were isolated from the blood by centrifugation for 15 minutes at 1500 to 1800 G. After centrifugation, the fraction containing PBMCs was transferred to a 15 mL conical centrifuge tube and the PBMCs were washed twice with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}. The final wash of the cell pellet was kept at -70°C until analysis.

Measurement of the prodrug, metabolite X and GS8373 in plasma and PBMCs

For plasma sample analysis, the samples were processed by a solid phase extraction (SPE) procedure outlined below. Speedisk C18 solid phase extraction cartridges (1 mL, 20 mg, 10 μM, from J.T. Baker) were conditioned with 200 μL of methanol followed by 200 μL of water. An aliquot of 200 μL of plasma sample was applied to each cartridge, followed by two washing steps each with 200 μL of deionized water. The compounds were eluted from the cartridges with a two-step process each with 125 μL of methanol. Each well was added 50 μL of water and mixed. An aliquot of 25 μL of the mixture was injected onto a ThermoFinnigan TSQ Quantum LC/MS/MS system.

The column used in liquid chromatography was HyPURITY® C18 (50 x 2.1 mm, 3.5 um) from Thermo-Hypersil. Mobile phase A contained 10% acetonitrile in 10 mM ammonium formate, pH 3.0. Mobile phase B contained 90% acetonitrile in 10 mM ammonium formate, pH 4.6. The chromatography was carried out at a flow rate of 250 μL/min under an isocratic condition of 40% mobile phase A and 60% mobile phase B. Selected reaction monitoring (SRM) were used to measure GS77366, GS8373 and Metabolite X with the positive ionization mode on the electrospray probe. The limit of quantitation (LOQ) was 1 nM for GS77366, GS8373 and GS77568 (Metabolite X) in plasma.

For PBMC sample analysis, phosphate buffered saline (PBS) was added to each PBMC pellet to bring the total sample volume to 500 μL in each sample. An aliquot of 150 μL from each PBMC sample was mixed with an equal volume of methanol, followed by the addition of 700 μL of 1% formic acid in water. The resulting mixture was applied to a Speedisk C18 solid phase extraction cartridge (1 mL, 20 mg, 10 um, from J.T. Baker) which had been conditioned as described above. The compounds were eluted with methanol after washing the cartridge 3 times with 10% methanol. The solvent was evaporated under a stream of N_2, and the sample was reconstituted in 150 μL of 30% methanol. An aliquot of 75 μL of the solution was injected for LC/MS/MS analysis. The limit of quantitation was 0.1 ng/mL in the PBMC suspension.

Pharmacokinetic Calculations

The pharmacokinetic parameters were calculated using WinNonlin. Noncompartmental analysis was used for all pharmacokinetic calculation. The intracellular concentrations in PBMCs were calculated from the measured concentrations in PBMC suspension on the basis of a reported volume of 0.2 picoliter/cell (B.L. Robins, R.V. Srinivas, C. Kim, N. Bischofberger, and A. Fridland, (1998) *Antimicrob. Agents Chemother.* 42, 612).

Plasma and PBMC Concentration-time Profiles

The concentration-time profiles of GS77366, GS77568 and GS8373 in plasma and PBMCs following intravenous dosing of GS77366 were compared at 1 mg/kg in dogs. The data demonstrate that the prodrug can effectively deliver the active components (metabolite X and GS8373) into cells that are primarily responsible for HIV replication, and that the active components in these cells had much longer half-life than in plasma.

The pharmacokinetic properties of GS77568 in PBMCs following oral administration of GS77366 in dogs are compared with that of nelfinavir and amprenavir, two marketed HIV protease inhibitors (Table 3). These data show that the active component (GS77568) from the phosphonate prodrug had sustained levels in PBMCs compared to nelfinavir and amprenavir.

Table 3: Comparison of GS77568 with nelfinavir and amprenavir in PBMCs following oral administration in beagle dogs.

Compound	Dose	$t_{1/2}$ (hr)	$AUC_{(2\text{-}24\ hr)}$
Nelfinavir	17.5 mg/kg	3.0 hr	33,000 nM·hr
Amprenavir	20 mg/kg	1.7 hr	102,000 nM·hr
GS77568	20 mg/kg of GS77366	> 20 hr	42,200 nM·hr

Intracellular Metabolism/*In Vitro* Stability

1. Uptake and Persistence in MT2 cells, quiescent and stimulated PBMC

The protease inhibitor (PI) phosphonate prodrugs undergo rapid cell uptake and metabolism to produce acid metabolites including the parent phosphonic acid. Due to the presence of charges, the acid metabolites are significantly more persistent in the cells than non-charged PI's. In order to estimate the relative intracellular levels of the different PI prodrugs, three compounds representative of three classes of phosphonate PI prodrugs – bisamidate phosphonate, monoamidate phenoxy phosphonate and monolactate phenoxy phosphonate (Figure 1) were incubated at 10 μM for 1 hr with MT-2 cells, stimulated and quiescent peripheral blood mononuclear cells (PBMC) (pulse phase). After incubation, the cells were washed, resuspended in the cell culture media and incubated for 24 hr (chase phase). At specific time points, the cells were washed, lysed and the lysates were analyzed by HPLC with UV detection. Typically, the cell lysates were centrifuged and 100 uL of the supernatant were mixed with 200 μL of 7.5 uM amprenavir (Internal Standard) in 80% acetonitrile/20% water and injected into an HPLC system (70 μL).

HPLC Conditions:

Analytical Column: Prodigy ODS-3, 75 x 4.6, 3u + C18 guard at 40°C Gradient:

Mobile Phase A: 20 mM ammonium acetate in 10% ACN/90% H_2O

Mobile Phase B: 20 mM ammonium acetate in 70% ACN/30% H_2O

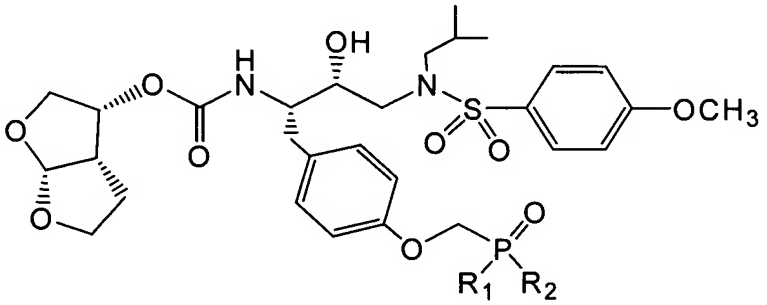
30-100%B in 4 min, 100%B for 2 min, 30%B for 2 min at 2.5 mL/min.

Run Time: 8 min

UV Detection at 245 nm

Concentrations of Intracellular metabolites were calculated based on cell volume 0.2 μL/mLn cells for PBMC and 0.338 μL / mLn (0.676 uL / mL) for MT-2 cells.

Table 4: Chemical Structures of Selected Protease Inhibitor Phosphonate Prodrugs and Intracellular Metabolites



GS No.	R1	R2	EC_{50} (nM)
8373	OH	OH	4,800±1,800
16503	$HNCH(CH_3)COOBu$	$HNCH(CH_3)COOBu$	6.0±1.4
16571	OPh	$HNCH(CH_3)COOEt$	15±5
17394	OPh	$OCH(CH_3)COOEt$	20±7
16576	OPh	$HNCH(CH_2CH_3)COOEt$	12.6±4.8
Met X	OH	$HNCH(CH_3)COOH$	>10,000
Met LX	OH	$OCH(CH_3)COOEt$	1750±354

A significant uptake and conversion of all 3 compounds in all cell types was observed (Table 4). The uptake in the quiescent PBMC was 2-3-fold greater than in the stimulated cells. GS-16503 and GS-16571 were metabolized to Metabolite X and GS-8373. GS-17394 metabolized to the Metabolite LX. Apparent intracellular half-lives were similar for all metabolites in all cell types (7-12 hr). A persistence of Total Acid Metabolites of Protease

Inhibitor Prodrugs in Stimulated (A), Quiescent PBMC (B) and MT-2 Cells (C) (1 hr, 10 uM Pulse, 24 hr Chase) was observed.

2. Uptake and Persistence in Stimulated and Quiescent T-cells

Since HIV mainly targets T-lymphocytes, it is important to establish the uptake, metabolism and persistence of the metabolites in the human T-cells. In order to estimate the relative intracellular levels of the different PI prodrugs, GS-16503, 16571 and 17394 were incubated at 10 μM for 1 hr with quiescent and stimulated T-cells (pulse phase). The prodrugs were compared with a non-prodrug PI, nelfinavir. After incubation, the cells were washed, resuspended in the cell culture media and incubated for 4 hr (chase phase). At specific time points, the cells were washed, lysed and the lysates were analyzed by HPLC with UV detection. The sample preparation and analysis were similar to the ones described for MT-2 cells, quiescent and stimulated PBMC.

Table 5 demonstrate the levels of total acid metabolites and corresponding prodrugs in T-cells following pulse/chase and continuous incubation. There was significant cell uptake/metabolism in T-lymphocytes. There was no apparent difference in uptake between stimulated and quiescent T-lymphocytes. There was significantly higher uptake of phosphonate PI's than nelfinavir. GS17394 demonstrates higher intracellular levels than GS16571 and GS16503. The degree of conversion to acid metabolites varied between different prodrugs. GS-17394 demonstrated the highest degree of conversion, followed by GS-16503 and GS-16571. The metabolites, generally, were an equal mixture of the mono-phosphonic acid metabolite and GS-8373 except for GS-17394, where Metabolite LX was stable, with no GS-8373 formed.

Table 5: Intracellular Levels of Metabolites and Intact Prodrug Following Continuous and 1 hr Pulse/4 hr Chase Incubation (10 μM/0.7 mLn cells/1 mL) of 10 μM PI Prodrugs and Nelfinavir with Quiescent and Stimulated T-cell

Compound	Time (h)	Continuous Incubation				1 hr Pulse /4 hr Chase			
		Quiescent T-cells		Stimulated T-cells		Quiescent T-cells		Stimulated T-cells	
		Acid Met (μM)	Prodrug (μM)	Acid Met (μM)	Prodrug (μM)	Acid Met (μM)	Prodrug (μM)	Acid Met (μM)	Prodrug (μM)
16503	0	1180	42	2278	0	2989	40	1323	139
	2	3170	88	1083	116	1867	4	1137	31
	4	5262	0	3198	31	1054	119	1008	0
16571	0	388	1392	187	1417	1042	181	858	218
	2	947	841	1895	807	1170	82	1006	35
	4	3518	464	6147	474	1176	37	616	25
17394	0	948	1155	186	1194	4480	14	2818	10
	2	7231	413	3748	471	2898	33	1083	51
	4	10153	167	3867	228	1548	39	943	104
Nelfinavir	0		101		86		886		1239
	2		856		846		725		770
	4		992		1526		171		544

3. PBMC Uptake and Metabolism of Selected PI Prodrugs Following 1-hr Incubation in MT-2 Cells at 10, 5 and 1 μM

To were similar to the determine if the cell uptake/metabolism is concentration dependent, selected PI's were incubated with the 1 mL of MT-2 cell suspension (2.74 mLn cells/mL) for 1 hr at 37°C at 3 different concentrations: 10, 5 and 1 μM. Following incubation, cells were washed twice with the cell culture medium, lysed and assayed using HPLC with UV detection. The sample preparation and analysis ones described for MT-2 cells, quiescent and stimulated PBMC. Intracellular concentrations were calculated based on cell count, a published single cell volume of 0.338 pl for MT-2 cells, and concentrations of analytes in cell lysates. Data are shown in Table 6.

Uptake of all three selected PI's in MT-2 cells appears to be concentration-independent in the 1-10 μM range. Metabolism (conversion to acid metabolites) appeared to be concentration-dependent for GS-16503 and GS-16577 (3-fold increase at 1 μM vs. 10 μM) but independent for GS-17394 (monolactate). Conversion from a respective metabolite X to GS-8373 was concentration-independent for both GS-16503 and GS-16577 (no conversion was observed for metabolite LX of GS-17394).

Table 6: Uptake and Metabolism of Selected PI Prodrugs Following 1-hr Incubation in MT-2 Cells at 10, 5 and 1 μM

Compound	Extracellular Concentration, μM	Cell-Assosiated Prodrug and Metabolites Concentration, μM				% Conversion to acid metabolites
		Metabolite X	GS8373	Prodrug	Total	
GS-17394	10	1358	0	635	1993	68
	5	916	0	449	1365	67
	1	196	0	63	260	76
GS-16576	10	478	238	2519	3235	22
	5	250	148	621	1043	40
	1	65	36	61	168	64
GS-16503	10	120	86	1506	1712	12
	5	58	60	579	697	17
	1	12	18	74	104	29

* For GS16576, Metabolite X is mono-aminobutyric acid

4. PBMC Uptake and Metabolism of Selected PI Prodrugs Following 1-hr Incubation in Human Whole Blood at 10 μM

In order to estimate the relative intracellular levels of the different PI prodrugs under conditions simulating the in vivo environment, compounds representative of three classes of phosphonate PI prodrugs – bisamidate phosphonate (GS-16503), monoamidate phenoxy phosphonate (GS-16571) and monolactate phenoxy phosphonate(GS-17394) were incubated at 10 μM for 1 hr with intact human whole blood at 37°C. After incubation, PBMC were isolated, then lysed and the lysates were analyzed by HPLC with UV detection. The results of analysis are shown in Table 7. There was significant cell uptake/metabolism following incubation in whole blood. There was no apparent difference in uptake between GS-16503 and GS-16571. GS-17394 demonstrated significantly higher intracellular levels than GS-16571 and GS-16503.

The degree of conversion to acid metabolites varies between different prodrugs after 1 hr incubation. GS-17394 demonstrated the highest degree of conversion, followed by GS-16503 and GS-16571 (Table 7). The metabolites, generally, were an equimolar mixture of the mono-phosphonic acid metabolite and GS-8373 (parent acid) except for GS-17394, where Metabolite LX was stable with no GS-8373 formed.

Table 7: PBMC Uptake and Metabolism of Selected PI Prodrugs Following 1-hr Incubation in Human Whole Blood at 10 μM (Mean ± SD, N=3)

GS#	Intracellular Prodrug and Metabolites Concentration, μM			Major Intracellular Metabolites
	Acid Metabolite	Prodrug, μM	Total, μM	
16503	279 ± 47	61 ± 40	340 ± 35	X , GS-8373
16571	319 ± 112	137 ± 62	432 ± 208	X, GS-8373
17394	629 ± 303	69 ± 85	698 ± 301	LX

* PBMC Intracellular Volume = 0.2 μL/mln

5. Distribution of PI Prodrugs in PBMC

In order to compare distribution and persistence of PI phosphonate prodrugs with those of non-prodrug PI's, GS-16503, GS-17394 and nelfinavir, were incubated at 10 μM for 1 hr with PBMC (pulse phase). After incubation, the cells were washed, resuspended in the cell culture media and incubated for 20 more hr (chase phase). At specific time points, the cells were washed and lysed. The cell cytosol was separated from membranes by centrifugation at 9000 xg. Both cytosol and membranes were extracted with acetonitrile and analyzed by HPLC with UV detection.

Table 8 shows the levels of total acid metabolites and corresponding prodrugs in the cytosol and membranes before and after the 22 hr chase. Both prodrugs exhibited complete conversion to the acid metabolites (GS-8373 and X for GS-16503 and LX for GS-17394, respectively). The levels of the acid metabolites of the PI phosphonate prodrugs in the cytosol fraction were 2-3-fold greater than those in the membrane fraction after the 1 hr pulse and 10-fold greater after the 22 hr chase. Nelfinavir was present only in the membrane fractions. The uptake of GS-17394 was about 3-fold greater than that of GS-16503 and 30-fold greater than nelfinavir. The metabolites were an equimolar mixture of metabolite X and GS-8373 (parent acid) for GS-16503 and only metabolite LX for GS-17394.

Table 8: Uptake and Cell Distribution of Metabolites and Intact Prodrugs Following Continuous and 1 hr Pulse/22 hr Chase Incubation of 10 μM PI Prodrugs and Nelfinavir with Quiescent PBMC

GS#	Cell Type	Fraction	Cell-Associated PI, pmol/mln cells			
			1 hr Pulse/ 0 hr Chase		1 hr Pulse/ 22 hr Chase	
			Acid Metabolites	Prodrug	Acid Metabolites	Prodrug
GS-16503	PBMC	Membrane	228	0	9	0
GS-16503	PBMC	Cytosol	390	0	130	0
GS-17394	PBMC	Membrane	335	0	26	0
GS-17394	PBMC	Cytosol	894	0	249	0
Nelfinavir	PBMC	Membrane		42		25
Nelfinavir	PBMC	Cytosol		0		0

Uptake and cell distribution of metabolites and intact prodrugs following 1 hr pulse/22 hr chase incubation of 10 μM PI prodrugs and Nelfinavir with quiescent PBMC were measured.

6. PBMC Extract/Dog Plasma/Human Serum Stability of Selected PI Prodrugs

The *in vitro* metabolism and stability of the PI phosphonate prodrugs were determined in PBMC extract, dog plasma and human serum (Table 9). Biological samples listed below (120 μL) were transferred into an 8-tube strip placed in the aluminum 37°C heating block/holder and incubated at 37°C for 5 min. Aliquots (2.5 μL) of solution containing 1 mM of test compounds in DMSO, were transferred to a clean 8-tube strip, placed in the aluminum 37°C heating block/holder. 60 μL aliquots of 80% acetonitrile/20% water containing 7.5 μM of amprenavir as an internal standard for HPLC analysis were placed into five 8-tube strips and kept on ice/refrigerated prior to use. An enzymatic reaction was started by adding 120 μL aliquots of a biological sample to the strip with the test compounds using a multichannel pipet. The strip was immediately vortex-mixed and the reaction mixture (20 μL) was sampled and transferred to the Internal Standard/ACN strip. The sample was considered the time-zero sample (actual time was 1-2 min). Then, at specific time points, the reaction mixture (20 μL) was sampled and transferred to the corresponding IS/ACN strip. Typical sampling times were 6, 20, 60 and 120

min. When all time points were sampled, an 80 μL aliquot of water was added to each tube and strips were centrifuged for 30 min at 3000xG. The supernatants were analyzed with HPLC under the following conditions:

Column: Inertsil ODS-3, 75 x 4.6 mm, 3 μm at 40°C.
Mobile Phase A: 20 mM ammonium acetate in 10%ACN/90%water
Mobile Phase B 20 mM ammonium acetate in 70%ACN/30%water
Gradient: 20% B to 100% B in 4 min, 2 min 100% B, 2 min 20% B
Flow Rate: 2 mL/min
Detection: UV at 243 nm
Run Time: 8 min

The biological samples evaluated were as follows:

PBMC cell extract was prepared from fresh cells using a modified published procedure (A. Pompon, I. Lefebvre, J-L. Imbach, S. Kahn, and D. Farquhar, *Antiviral Chemistry & Chemotherapy*, 5, 91 - 98 (1994)). Briefly, the extract was prepared as following: The cells were separated from their culture medium by centrifugation (1000 g, 15 min, ambient temperature). The residue (about 100 μL, 3.5×10^8 cells) was resuspended in 4 mL of a buffer (0.010 M HEPES, pH 7.4, 50 mM potassium chloride, 5 mM magnesium chloride and 5 mM dl-dithiothreitol) and sonicated. The lysate was centrifuged (9000 g, 10 min, 4°C) to remove membranes. The upper layer (0.5 mg protein/mL) was stored at –70°C. The reaction mixture contained the cell extract at about 0.5 mg protein/mL.

Human serum (pooled normal human serum from George King Biomedical Systems, Inc.). Protein concentration in the reaction mixture was about 60 mg protein/mL.

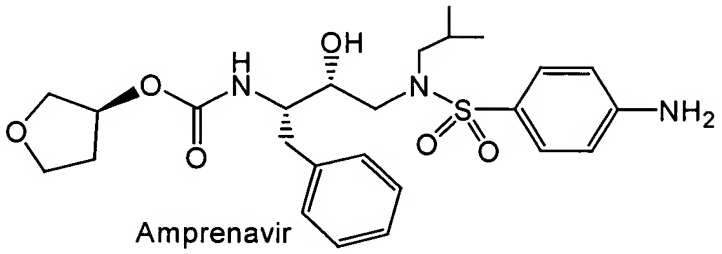
Dog Plasma (pooled normal dog plasma (EDTA) from Pel Freez, Inc.). Protein concentration in the reaction mixture was about 60 mg protein/mL.

Table 9: PBMC Extract/Dog Plasma/Human Serum Stability of Selected PI Prodrugs

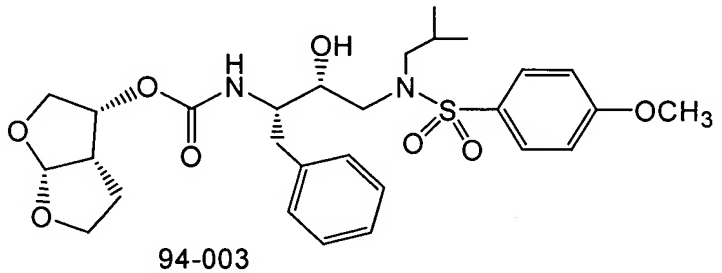
GS#	PBMC Extract[1] $T_{1/2}$, min	Dog Plasma $T_{1/2}$, min	Human Serum $T_{1/2}$, min	HIV EC_{50} (nM)
16503	2	368	>>400	6.0 ± 1.4
16571	49	126	110	15 ± 5
17394	15	144	49	20 ± 7

Table 10: Enzymatic and Cellular data

Formula II ALPPI activity



Amprenavir



94-003

Ki [pM]

≤ 10	+++
> 10 to ≤ 100	++
> 100 to ≤ 1,000	+
> 1,000	-

EC_{50} [nM]

≤ 50	+++
> 50 to ≤ 500	++
> 500 to ≤ 5,000	+
> 5,000	-

I50V and I84V/L90M fold change

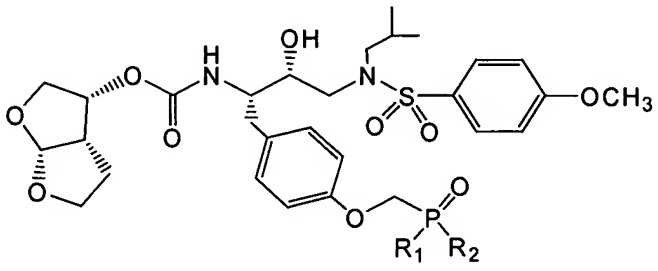
> 30	+++
> 10 to ≤ 30	++
> 3 to ≤ 10	+
≤ 3	-

CC_{50} [μM]

≤ 5	++
> 5 to ≤ 50	+
> 50	-

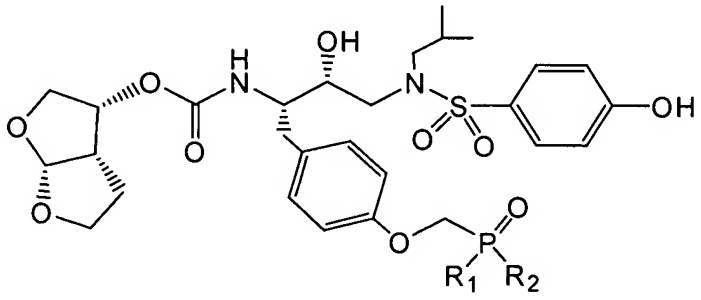
Compound	Ki (pM)	EC_{50} (nM)	I50V (#1) foldchange	I50V (#2) fold change	I84V/L90M fold change	CC_{50} (μM)
Saquinavir	++	+++	–	–	+++	
Nelfinavir	+	+++	–	+	+++	
Indinavir	+	+++	-	+	+++	
Ritonavir	++	+++	++	++	+++	
Lopinavir	++	+++	++	+++	++	
Amprenavir	+	+++	+++	+++	++	–
Atazanavir	++	+++	–	–	+++	
Tipranavir	++	++	–	–	+	
94-003	+++	+++	+++	+++	++	+
TMC114	+++	+++	++	++	–	

P1-Phosphonic acid and esters



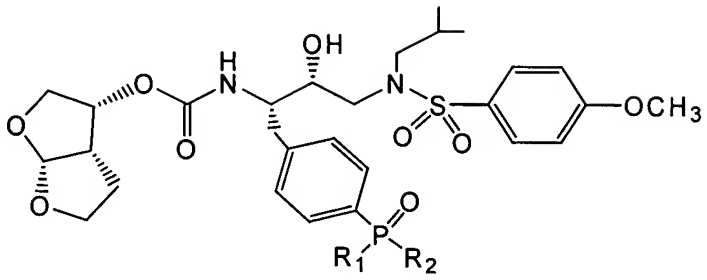
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} (μM)
OH	OH	+++	+	–	–	–
OMe	OMe	++	+++			
OEt	OEt	+++	+++	–	–	+
OCH_2CF_3	OCH_2CF_3	++	–			
OiPr	OiPr	++	+++	–	–	
OPh	OPh		+++			
OMe	OPh	++	+++			
OEt	OPh	+++	+++			
OBn	OBn	++	+++	–	–	+
OEt	OBn	++	+++			++
OPoc	OPoc		+			
OH	OEt		++			
OH	OPh	+++	–			
OH	OBn		+	–	–	

P1-Phosphonic acid and esters



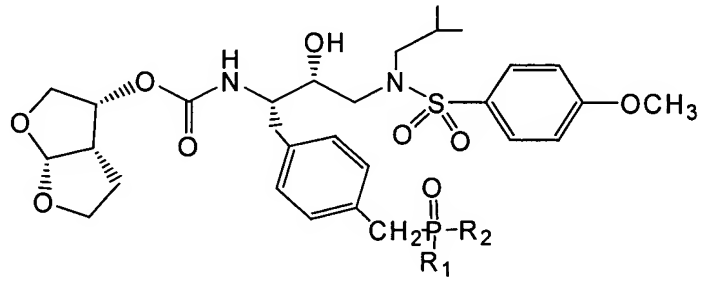
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} (μM)
OH	OH	+++	+			
Et	Et	+++	+++			

P1-Direct phosphonic acid and esters



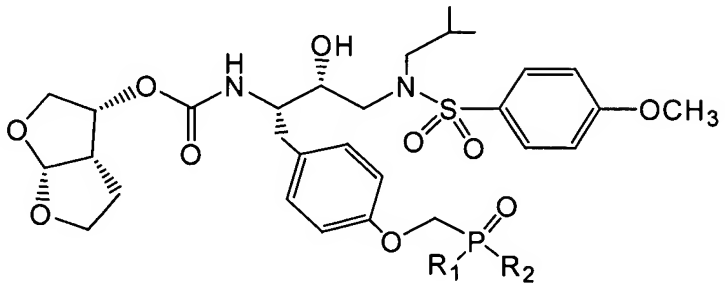
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
OH	OH	++	–			
OEt	OEt	+++	+++	+	–	

P1-CH_2-phosphonic acid and esters



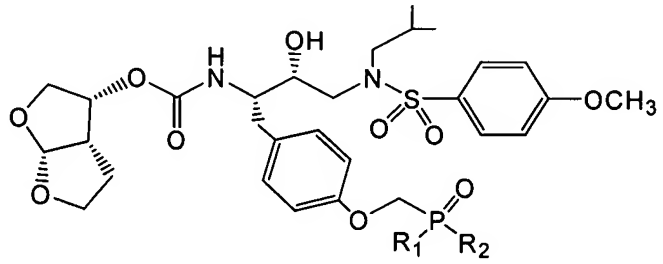
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
OE	OE	+++	+++	+	+	

P1-P-Bisamidates



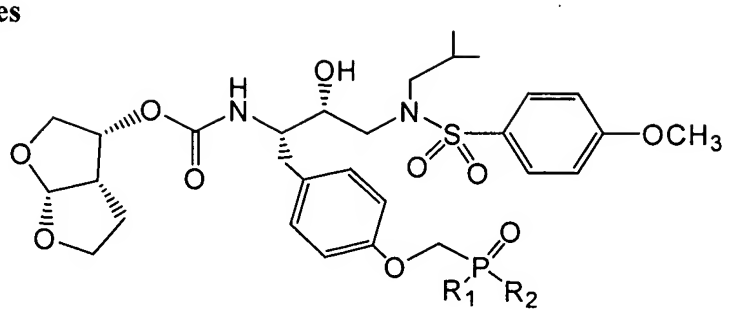
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
NHEt	NHEt	+++	++	–	–	
Gly-Et	Gly-Et	++	++			
Gly-Bu	Gly-Bu	+++	+++			
Ala-Et	Ala-Et	++	++		–	–
Ala-Bu	Ala-Bu	++	+++	+	–	
Aba-Et	Aba-Et	+++	+++			
Aba-Bu	Aba-Bu	+++	+++	++	+	
Val-Et	Val-Et	+	+++	–	–	
Leu-Et	Leu-Et	++	+++			
Leu-Bu	Leu-Bu	++	++	+	+	
Phe-Et	Phe-Et		+++			
Phe-Bu	Phe-Bu		+++			

P1-P-Bislactates



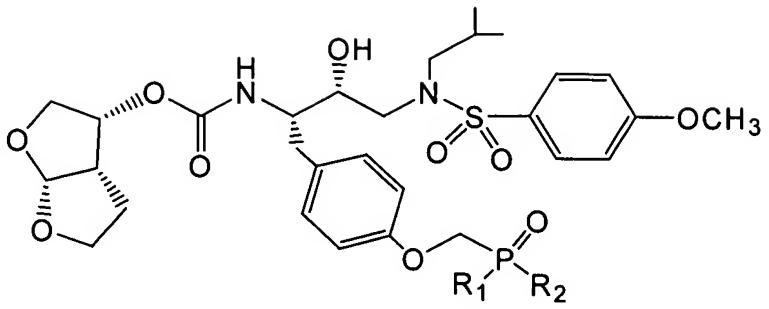
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
Glc-Et	Glc-Et	+++	+	–	–	
Lac-Et	Lac-Et	++	++	–	–	
Lac-iPr	Lac-iPr	++	+++		–	

P1-P-Monoamidates



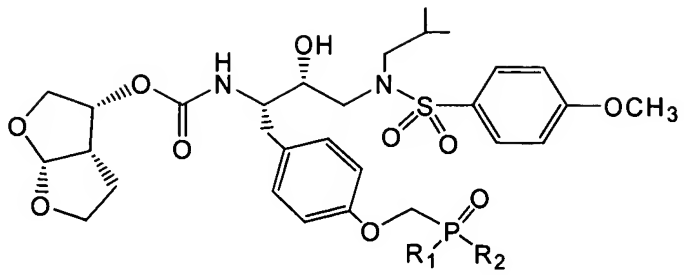
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
OPh	Gly-Bu	++	++	–	–	
OPh	Ala-Me	++	+++		–	
OPh	Ala-Et	+++	+++	–	–	
OPh	Ala-iPr	++	+++	–	–	
OPh	Ala-iPr	+++	+++			
OPh	Ala-iPr	++	+++			
OPh	(D)Ala-iPr	++	+++		–	
OPh	(D)Ala-iPr	+++	+++			
OPh	(D)Ala-iPr	+++	+++			
OPh	Ala-Bu	++	+++	–	–	
OPh	Ala-Bu	++	+++	–		
OPh	Ala-Bu	++	+++	–		
OPh	Aba-Et		+++			
OPh	Aba-Et		+++	–	–	
OPh	Aba-Et		++			
OPh	Aba-Bu		+++	+	–	
OPh	Aba-Bu		++	–	–	
OBn	Ala-Et	+++	+++	–	–	
OH	Ala-OH	+++	–			
OH	Ala-Bu		–			

P1-P-Monolactates (1)



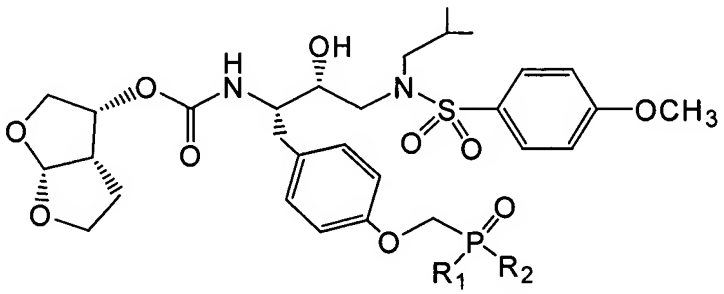
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I50V (#2) fold change	I84V/L90M fold change	CC_{50} μM
OPh	Glc-Et	+++	+++	–		–	
OPh	Lac-Me		++	–			
OPh	Lac-Et		+++	–	+	–	+
OPh	Lac-Et	+++	+++	–		–	
OPh	Lac-Et	++	+++	–		–	
OPh	Lac-iPr	++	+++	–		–	
OPh	Lac-iPr	+++	+++				
OPh	Lac-iPr	++	+++				
OPh	Lac-Bu	++	++			–	
OPh	Lac-Bu	++	++				
OPh	Lac-Bu	++	++				
OPh	Lac-EtMor		–				
OPh	Lac-PrMor		–				
OPh	(R)Lac-Me	+++	+++				
OPh	(R)Lac-Et	+++	+++	–		–	
OEt	Lac-Et		++				
OCH_2CF_3	Lac-Et		++				
OBn	Lac-Bn	++	++				
OBn	(R)Lac-Bn						
OH	Lac-OH	+++	+			–	
OH	(R)Lac-OH	++	+			–	

P1-P-Monolactates (2)



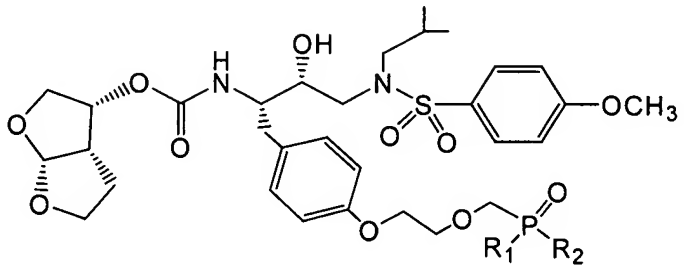
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
OPh	mix-Hba-Et	++	+++	+	–	
OPh	(S)Hba-Et	+	+++			
OPh	(S)Hba-tBu		+++			
OH	(S)Hba-OH	++				
OPh	(R)Hba-Et		+++			
OPh	(S)MeBut-Et		+++			
OPh	(R)MeBut-Et		+++			
OPh	DiMePro-Me	++				
OPh	(S)Lac-EtMor		–			
OPh	(S)Lac-PrMor		–			
OPh	(S)Lac-EtPip		++	–	–	

P1-P-Monolactates (3)



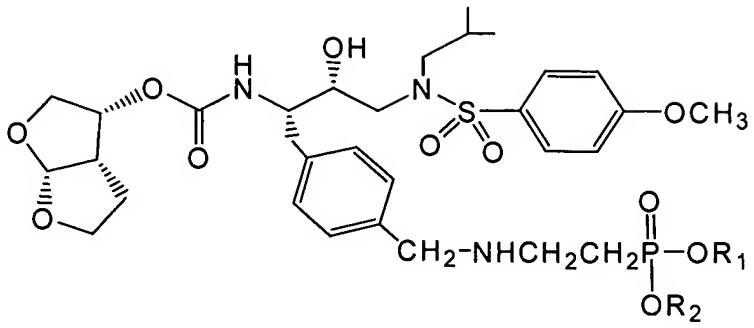
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
OPh—*o*-i-But	(S)Lac-Et		+++			
OPh—*p*-n-Oct	(S)Lac-Et		++			
OPh—*p*-n-But	(S)Lac-Et		+++			
OPh-*m*-COOBn	(S)Lac-Et		++			
OPh-*m*-COOH	(S)Lac-Et		++			
OPh-*m*-CH_2OH	(S)Lac-Et		++	–	–	
OPh-*m*-CH_2NH_2	(S)Lac-Et	++	++			
OPh-*m*-CH_2NMe_2	(S)Lac-Et		+			
OPh-*m*-CH_2Mor	(S)Lac-Et		++	–	–	
OPh-*m*-CH_2Pip	(S)Lac-Et		++			
OPh-*m*-CH_2NMeC2OMe	(S)Lac-Et		++			
Oph-*o*-OEt	(S)Lac-Et		+++			
$ONMe_2$	(S)Lac-Et		++			
OPip	(S)Lac-Et		+			
OMor	(S)Lac-Et		–			

P1-C_2H_4-P-Monolactates



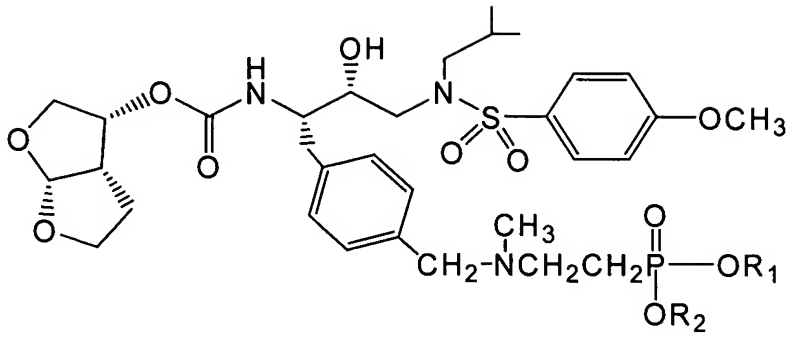
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
-OC_2H_4OBn			+++			
OEt	OEt		+++	–	–	
OPh	Lac-Et		++	–	–	
OH	OH	++				
OH	Lac	++				

P1-CH_2N-P-diester and monolactate (1)



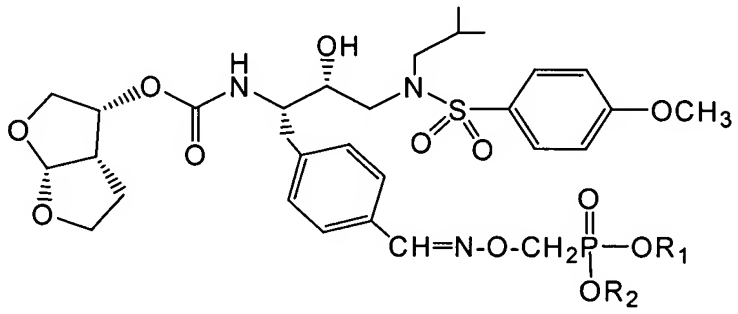
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I50V (#2) fold change	I84V/L9M fold change	CC_{50} µM
Et	Et	++	+++		–		
H	H	++	-		+		
Ph	Lac-Et		++	–	++	–	
Ph	Lac-Et		+		+	–	–
Ph	Lac-Et		+		++	–	
Ph	Aba-Et		+		+	–	
Ph-oEt	Lac-Et	++	++	–	++	–	
Ph-dM	Lac-Et		+++		+	+	
Ph-dM	Lac-Pr		+++				
H	Lac	++					
Ph	Hba-Et		++		++	–	
Ph	Hba-Et		++		++	–	+
Ph	Hba-Et		++		++	–	
H	Hba	+					

P1-CH_2N-P-diester and monolactate (2)



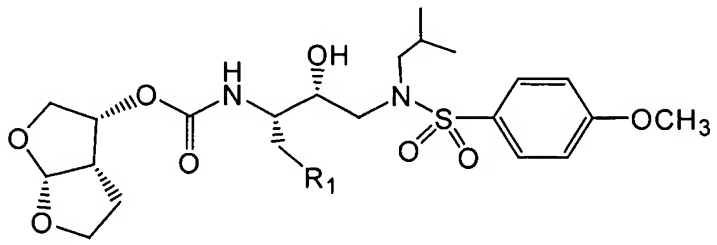
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
Ph	Lac-Et	+	++	+	+	
H	H	++				

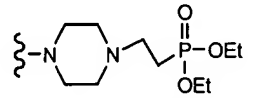
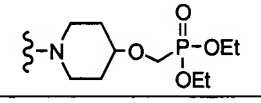
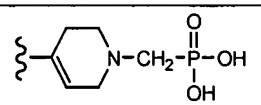
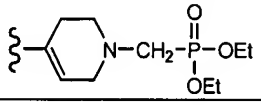
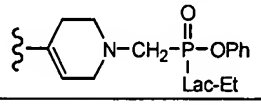
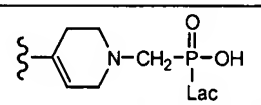
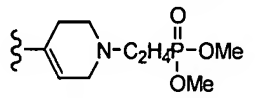
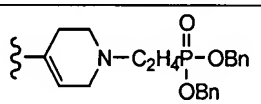
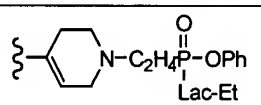
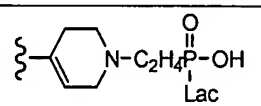
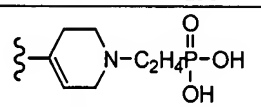
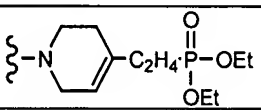
P1-CH_2N-P-diester and monolactate (3)



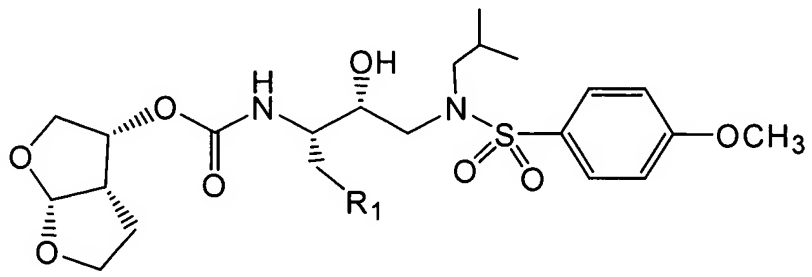
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
Et	Et	++	+++		–	

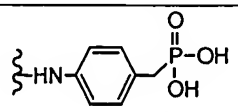
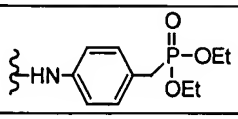
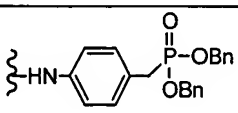
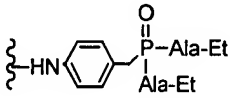
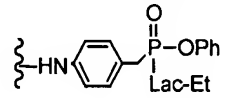
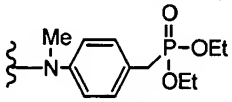
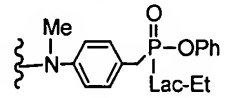
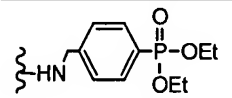
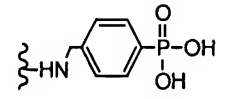
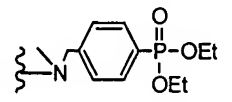
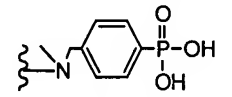
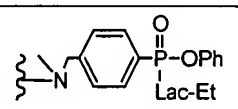
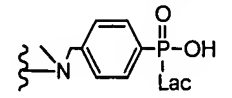
P1-N-P1-Phosphonic acid and esters (1)



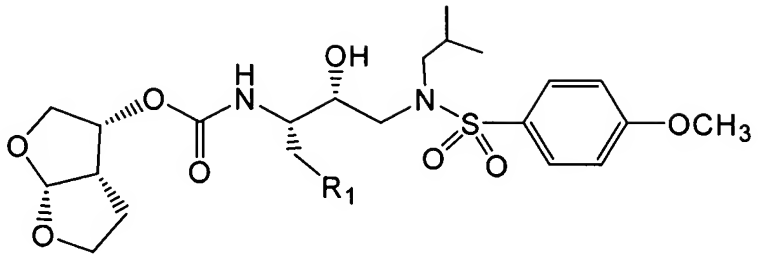
R1	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
	–	++			
	–	++			
	–				
	++	+++		+	
		–			
	–				
	+	++			
	++	+++		+	
		–			
		–			
	–				
	+	+++		+	

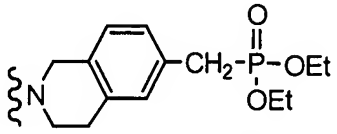
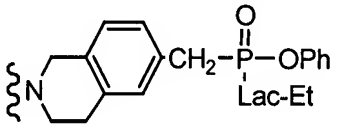
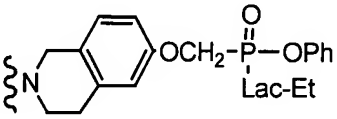
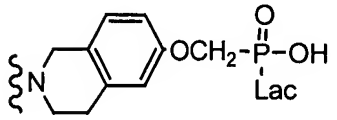
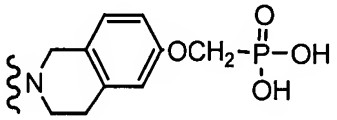
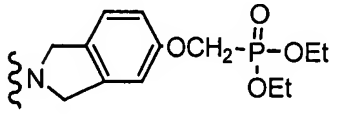
P1-N-P1-Phosphonic acid and esters (2)



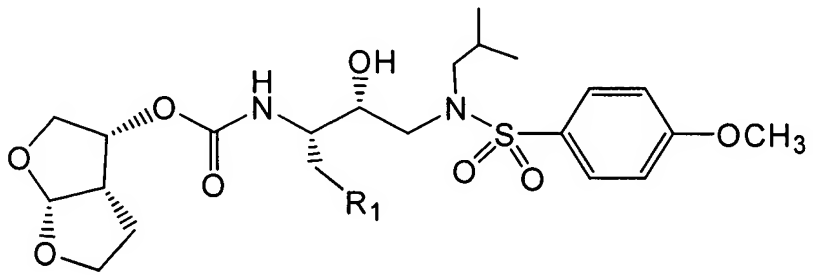
R1	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
	+	+		+	
	++	+++		+	
	++	+++			
	++	++		–	
		+++			
	++	+++		+	
		+++		–	
	–	+++		++	
	–				
	+	+++	+++	–	
	–				
		+++	++	+	
	–				

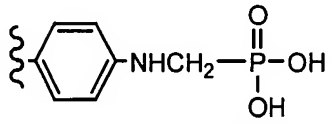
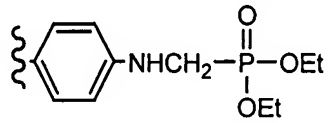
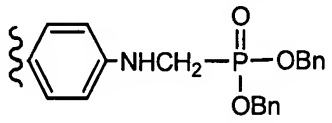
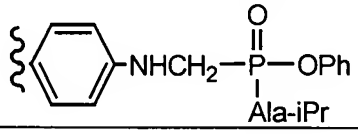
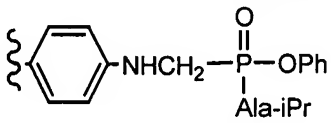
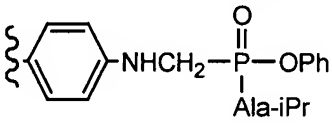
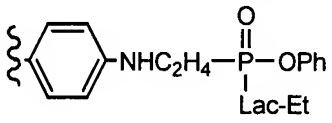
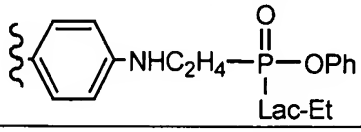
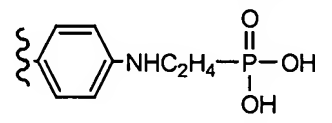
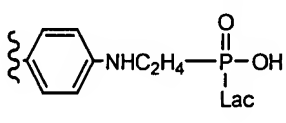
P1-N-P1-Phosphonic acid and esters (3)



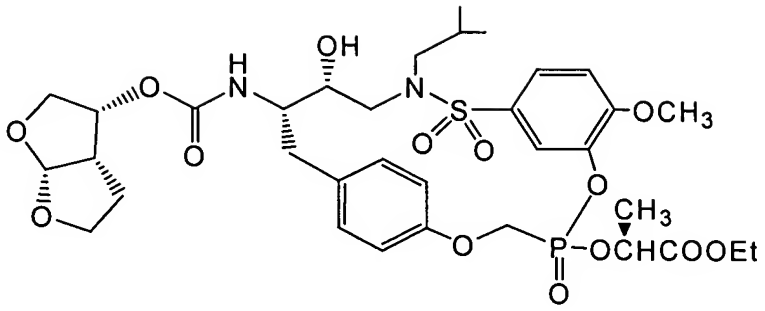
R1	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
	++	+++	+	+	
	+	++	+	+	
	+	++	+	+	
	+				
					
	–	–			

P1-N-P1-Phosphonic acid and esters (4)



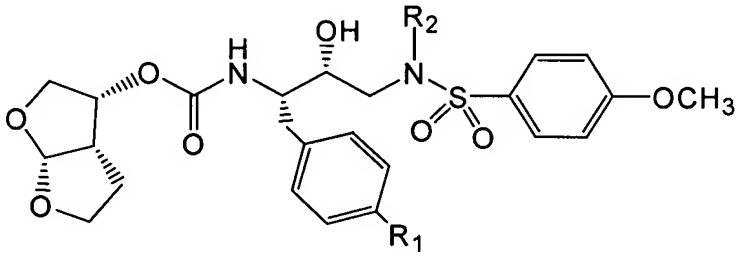
R1	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
	+++				
	+++	+++	–	–	
	++	+++	+	–	
	++	+++			
	++	++			
	+++	+++			
		+++	++	–	
		+++	++	–	
	++				
	++				

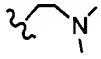
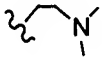
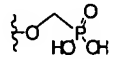
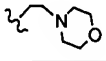
P1-P-cyclic monolactate

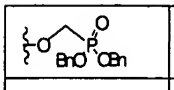
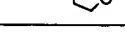
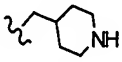
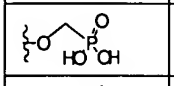
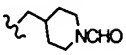
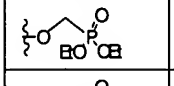
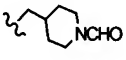
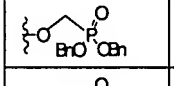
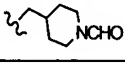
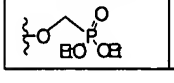
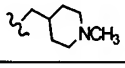


R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
		nd	nd			
		nd	nd			

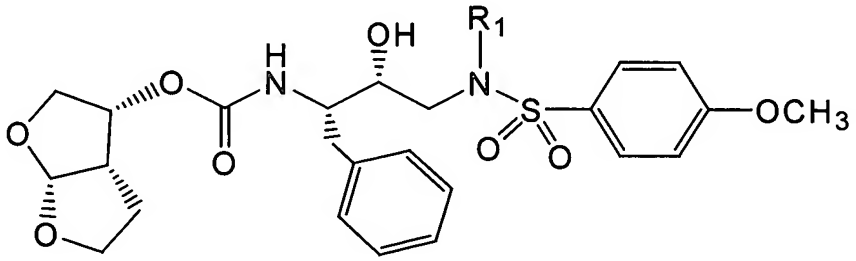
P1'-N-P1-Phosphonic acid and esters

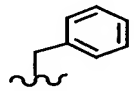
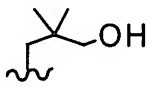
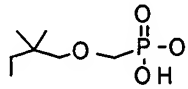
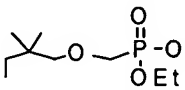
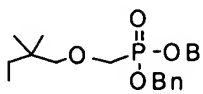
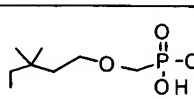
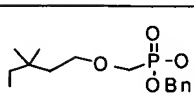


R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
CH_3		++	+++	++	+	
OH			+++	–	–	
CH_2OH		+++	+++	–	–	
OBn		+++	+++	–	–	
OH		–	++	–	–	
OBn		–	+++		–	
		–	–	+	+	

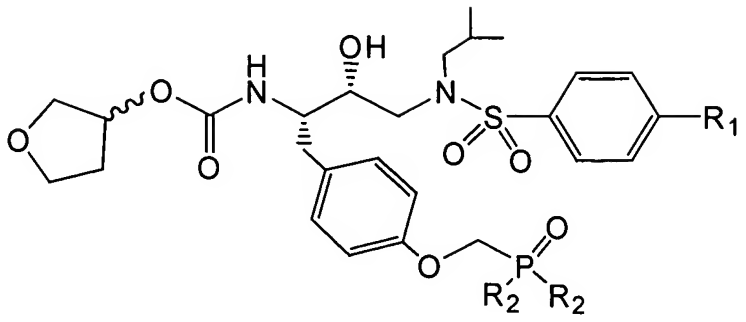
		+	++	+	+	
OH		–	–			
		++	–			
		++	–			
		++	++			
		+	–			

P1'-Phosphonic acid and esters



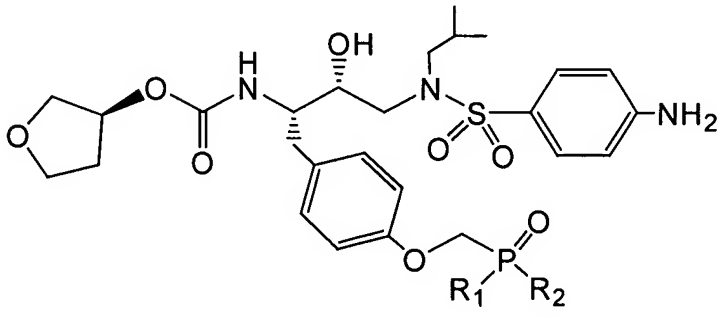
R1	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
	++	+++	+++	+++	
	+++	+++	+++	+++	
	++	+		+++	
	+++	+++		+++	
	+++	+++		++	
	++	++	++	++	
	++	+++	+++	+++	

P2-Monofuran-P1-phosphonic acid and esters



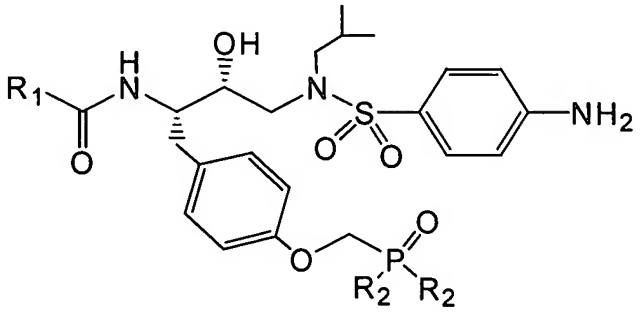
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
OMe	OH		–	+++	+++	
OMe	OEt	+++	+++	+++	++	
OMe	OBn		+++	++	++	
OMe	phenol	+++	+++	+++	+	
OMe	OEt	++	+++	+++	++	
NH_2	phenol	+	++	+	–	
NH_2	OH		–		+	
NH_2	OBn	++	++		+	

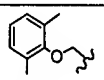
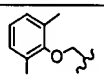
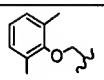
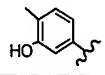
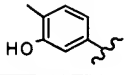
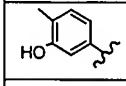
P2-Monofuran-P1-P-monoamidates



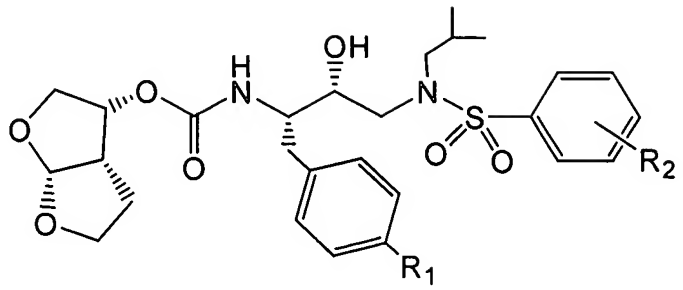
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
OPh	Ala-iPr	++	++		+	
OPh	Ala-iPr	++	++			
OPh	Ala-iPr	+	++			

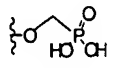
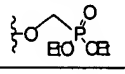
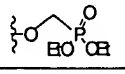
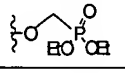
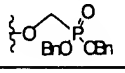
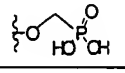
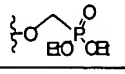
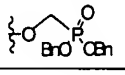
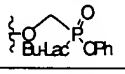
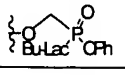
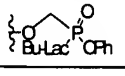
P2-Other modifications-P1-phosphonic acid and esters



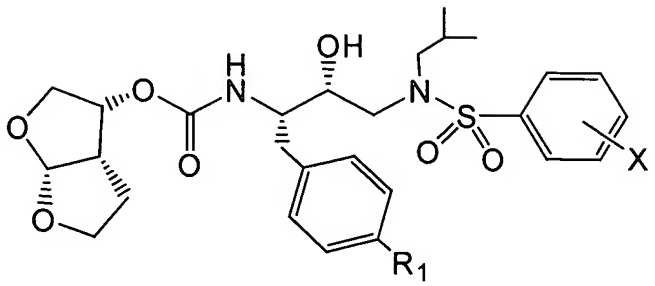
R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
	phenyl	+	+++	+++	++	
	phenol	+	++	++	+	
	OH	–	–	++	–	
	OBn	+	++	+	–	
	phenyl	+	++	+++	+	
	OH	+	–	++	+	
	OBn	+	++	+++	+	
	phenyl	–	++		++	
	phenol	+	+		–	
	OH	+	–	–	–	
	OBn	++	++	+	–	

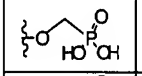
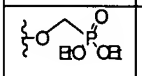
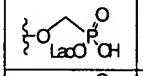
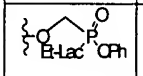
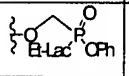
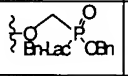
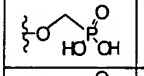
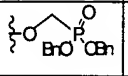
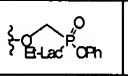
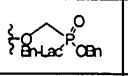
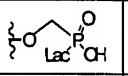
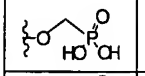
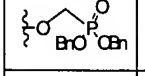
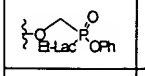
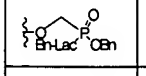
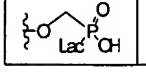
P2'-Amino-P1-phosphonic acid and esters

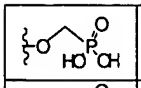
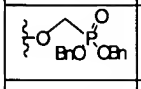
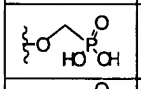
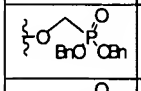
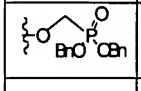
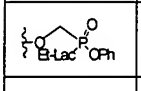
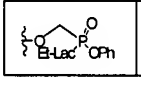


R1	R2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
OH	*p*-NH_2	++	++	–	–	
	*p*-NH_2	++	–	+	–	
	*p*-NH_2	++	+++		–	
	*p*-NO_2	++	+++		–	
	*p*-NHEt	++	+++		–	
	*p*-NH_2	++	+++	–	–	
OH	*m*-NH_2	++	++		–	
	*m*-NH_2	++	+		–	
	*m*-NH_2	++	++		–	
	*m*-NH_2	++	+++	–	–	
	*m*-NH_2	+	++	–	–	
	*m*-NH_2	++	++			
	*m*-NH_2	+	++			

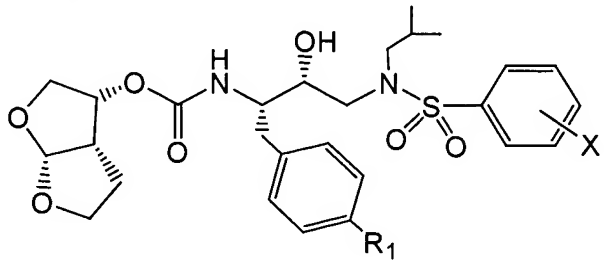
P2'-Substituted-P1-phosphonic acid and esters (1)

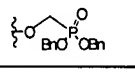
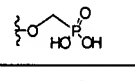
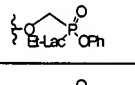
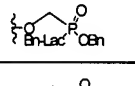
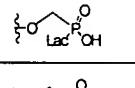
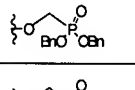
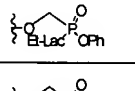
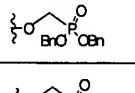
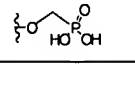


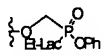
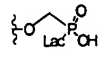
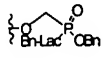
R1	X	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
	*p*-OH	+++	+			
	*p*-OH	+++	+++			
	*p*-OH	++				
	*p*-OH		+++		–	
	*p*-OBn		++			
	*p*-OBn		–			
	*p*-H	++	–			
	*p*-H	++	+++		+	
	*p*-H		+++	+	+	
	*p*-H		++			
	*p*-H	++				
	*p*-F	++	+			
	*p*-F	++	+++		+	
	*p*-F		+++	+	+	
	*p*-F		++	+	+	
	*p*-F	++				

	*p*-CF_3	+++	+			
	*p*-CF_3	++	+++		–	
	*p*-OCF_3	++	+			
	*p*-OCF_3	++	+++		+	
	*p*-CN	++	+++		–	
	*p*-Pip	–	–			
	*p*-Pip-Me	–	–			

P2'-Substituted-P1-phosphonic acid and esters (2)



R1	X	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
	*m*-Py	++	+++			
	*m*-Py	++				
	*m*-Py	++	++	+	–	
	*m*-Py	++	++			
	*m*-Py	++				
	*m*-Py-Me^+		+			
	*m*-Py-Me^+		++			
	*m*-Py-oxide		++			
	*m*-Py-oxide	++				

	*m*-Py-oxide	++	++		–	
	*m*-Py-oxide	+				
	*m*-Py-oxide		–			
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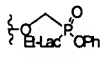
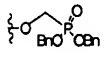
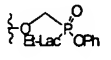
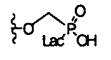
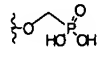
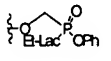
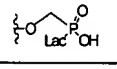
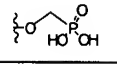
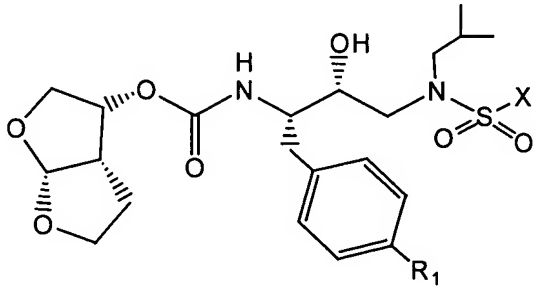
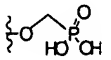
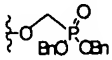
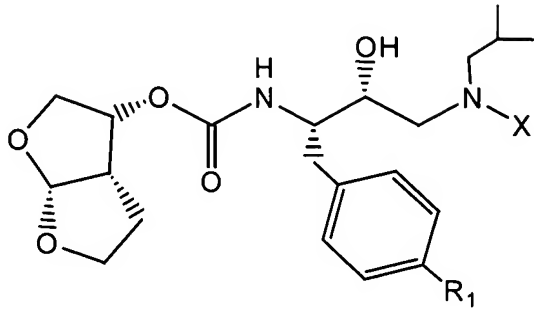
	*p*-CHO		+++			
	*p*-CHO		+++			
	*p*-CH2 OH		+++	–	–	
	*p*-CH2 OH	++				
	*p*-CH2 OH	++				
	*p*-CH2 Mor		++	–	–	
	*p*-CH2 Mor	–				
	*p*-CH2 Mor	–				

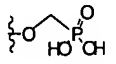
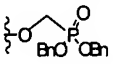
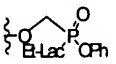
P2'-Alkylsulfonyl-P1-phosphonic acid and esters



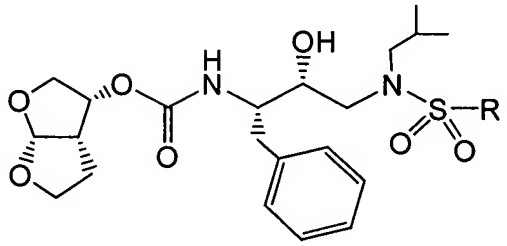
R1	X	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
		–	–			
		+	++			

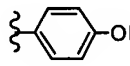
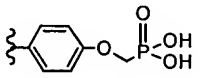
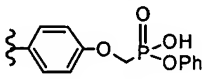
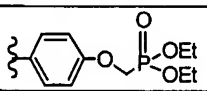
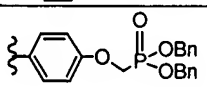
P2'-Carbonyl-substituted-P1-phosphonic acid and esters

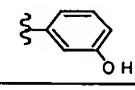
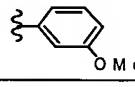
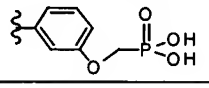
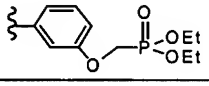
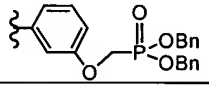
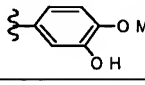
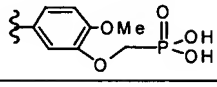
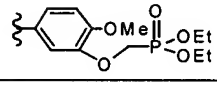
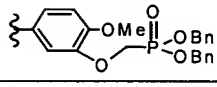
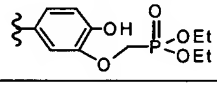


R1	X	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
		–				
		–	++			
			+			

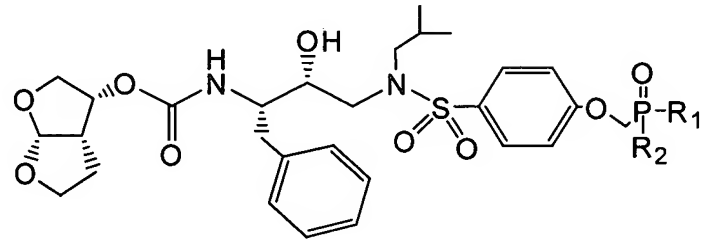
P2'-Phosphonic acid and esters



R	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
	+++	+++	–	–	
	+++	+	–	–	
	++	–			
	++	+++	++	++	
	+	++	+++	+++	

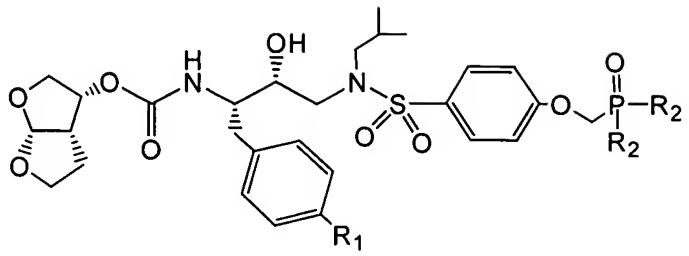
	+++	+++	+	+	
	+++	+++	+++	++	
	++	++	++	+	
	+++	+++	+++	++	
	++	+++	++	++	
	+++	+++	–	–	
	+++	++	+	–	
	+	++	+	+	
	–	+	+++	++	
	+	++	+	–	

P2'-P-Bisamidate, monoamidate, and monolactate



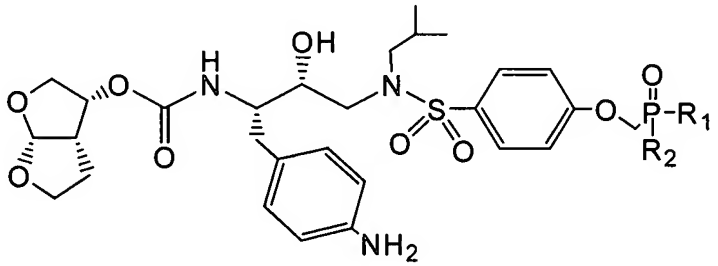
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
Ala-Bu	Ala-Bu	+	++	+	+	
OPh	Ala-iPr	++	++			
OPh	Lac-iPr	+	+			
OH	Ala-OH	++				

P1-N-P2'-Phosphonic acid and esters



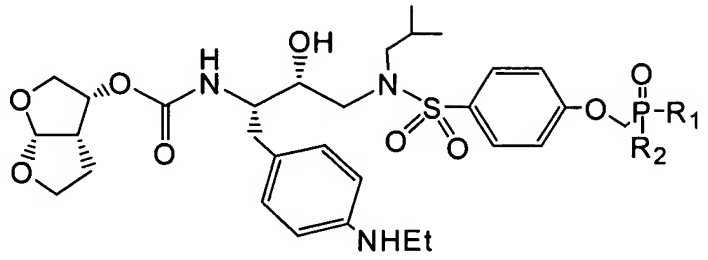
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
NO_2	phenol		+++	–		
NH_2	OH	++	-			
NH_2	OEt	+	++		++	
NH_2	OBn	+	+		+	
NMe_2	OEt	++	+++		++	
OH	OH	++	-			
OH	OBn	++	++			
$OC_2H_4NMe_2$	OH	+++	+			
OC_2H_4-NMe_2	OBn	++	++			

P1-N-P2'-P-Bisamidate and monoamidate



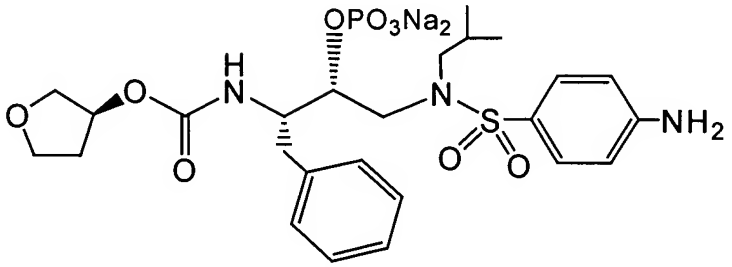
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
Ala-Bu	Ala-Bu	+	+			
OPh	Ala-iPr	+	–			
OPh	Ala-iPr	++	–			

P1-NEt-P2'-P-Bisamidate and monoamidate



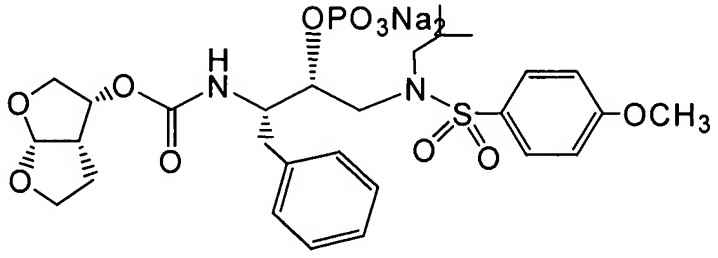
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
OPh	Ala-iPr	+	+			
OPh	Ala-iPr	+	+	–	–	

Phosphate prodrug of ampenavir



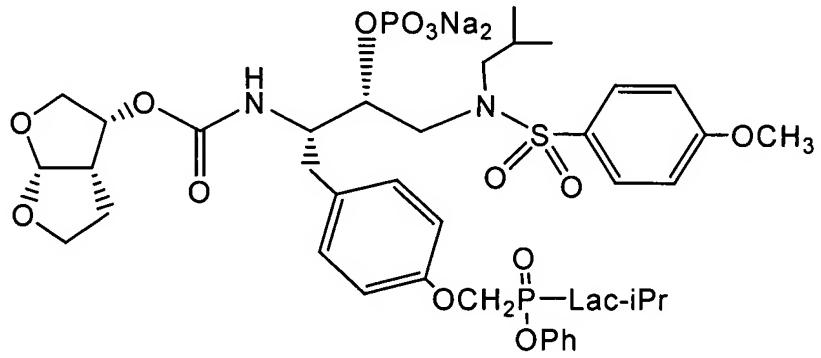
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
			++			

Phosphate prodrug of 94-003



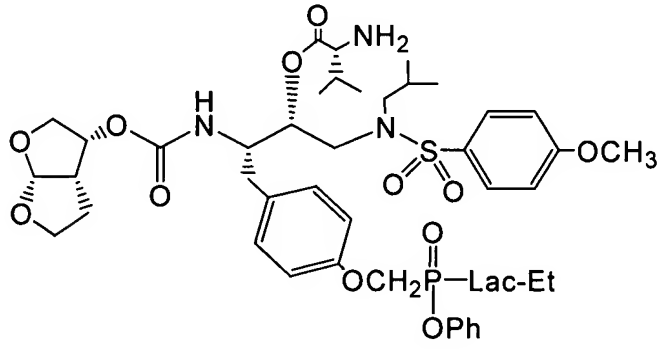
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
			+++			

Phosphate prodrug of GS77366 (P1-mono(S)Lac-iPr)



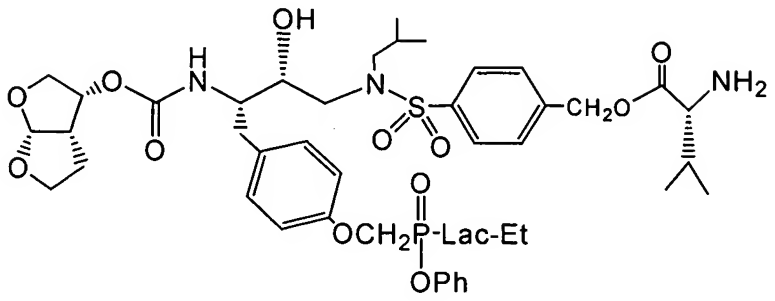
R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
			+++			

Valine prodrug of (P1-mono(S)Lac-Et)



R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} µM
			++			

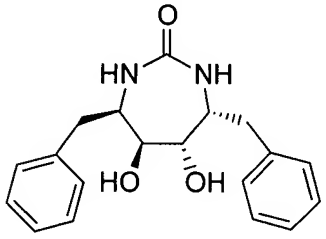
Valine prodrug of GS278053 (P1-mono(S)Lac-Et,P2'-CH_2OH)

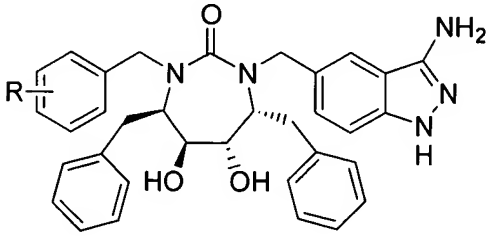


R_1	R_2	Ki (pM)	EC_{50} (nM)	I50V (#1) fold change	I84V/L90M fold change	CC_{50} μM
			++			

Table 11: Enzymatic and Cellular Activity Data

Formula VIIIa CCLPPI activity

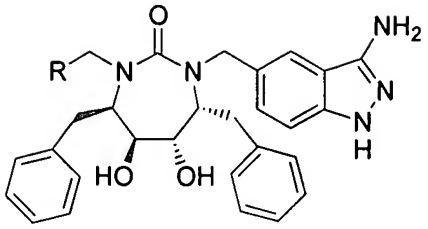


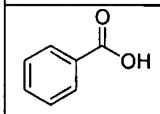
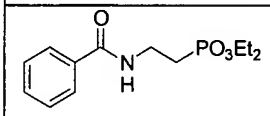
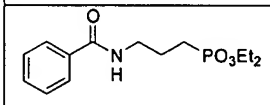
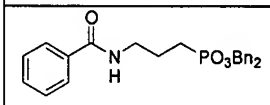
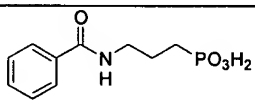
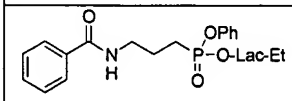
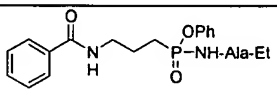


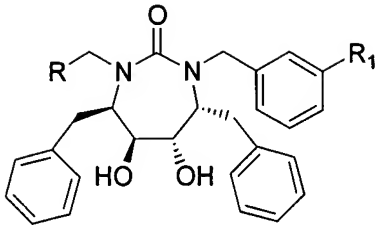
DMP-850

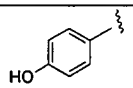
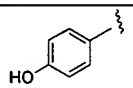
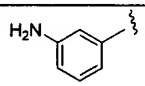
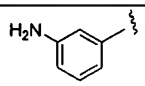
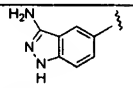
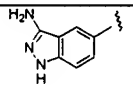
	Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
Structure, R	K_i (nM)	WT IC_{50} / nM	84V 90M IC_{50} / nM	WT	84V90M	30N 82I8D	48V 54V 82A	48V 54V 82S	48V 82A 90M	46I 50V
H (DMP-850)	0.033	3.0	9.1	165	819	82	82	73	45	88
p-OH	0.029	3.0	12	149	143	79	32	39	19	55
p-OBn	>5	353	781	2123	5312	1548	ND	ND	ND	ND
p-$OCH_2PO_3Bn_2$	>5	276	2042	2697	4963	2119	ND	ND	ND	ND

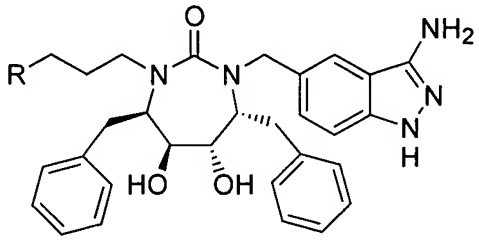
	Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
Structure, R	K_i (nM)	WT IC_{50} / nM	84V 90M IC_{50} / nM	WT	84V90M	30N 82I8 8D	48V 54V 82A	48V 54V 82S	48V 82A 90M	46I 50V
p-$OCH_2PO_3Et_2$	>5	627	1474	2480	>6000	1340	ND	ND	ND	ND
p-$OCH_2PO_3H_2$	>5	551	1657	>12000	ND	ND	ND	ND	ND	ND
m-OH	0.128	1.6	12	151	475	249	84			104
m-OBn	0.253	6.9	27	218	2422	82	709	ND	ND	601
m-$OCH_2PO_3Bn_2$ (N-iPr indazole)	1.54[a]	31	72	489	514	237	159	171	168	708
m-$OCH_2PO_3Bn_2$	0.177	18	43	898	>6000	705	2597	ND	ND	3121
m-$OCH_2PO_3Et_2$	1.93[a]	70	169	665	3005	93	513	ND	ND	857
m-$OCH_2PO_3H_2$	0.254	8.3	33	>12000	ND	ND	ND	ND	ND	ND
m-$OCH_2PO_3Ph_2$	0.543[a]	10	42	1349	>6000	1541	2183	ND	ND	3380
m-OCH_2PO_3HPh	0.644	17	65	1745	>6000	ND	ND	ND	ND	ND
m-mono-Ala-Bu	0.858[a]	6.6	39	1042	>6000	425	790	ND	ND	797
m-mono-Ala-Et[¶]		35	68	1436	>6000	219	734	ND	ND	1350
m-mono-Lac-Bu		15	34	2663	>6000	1089	ND	ND	ND	ND
m-mono-Lac-Et		23	80	2609	>6000	516	5923	ND	ND	>6000
m-bis-Ala-Bu	1.279[a]	18	103	1079	>6000	2362	1854	ND	ND	1536
m-bis-Ala-Et	1.987[a]	31	202	5620	>6000	1852	ND	ND	ND	ND



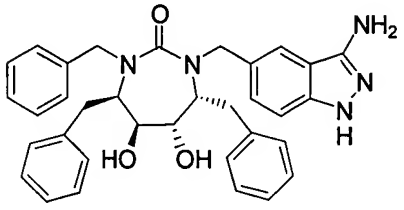
	Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
Structure, R	K_i (nM)	WT IC_{50}/ nM	84V 90M IC_{50} / nM	WT	84V90 M	30N 82I88 D	48V 54V 82A	48V 54V 82S	48V 82A 90 M	46I5 0V
H (DMP-850)	0.033	3.0	9.1	165	819	82	82	73	45	88
	0.091	3.4	27	1548	>6000	>6000	ND	ND	ND	ND
	0.354	3.3	25	168	909	750	277			489
	0.157	1.6	10	188	476	666	240			319
	0.044	5.0	27	491	387	234	238			192
	0.362	7.3	70	5141	>6000	4480	ND	ND	ND	ND
	0.112	1.4	6.4	603	1276	678	208			209
	<0.03	1.3	7.5	625	708	899	301			398



		Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
Structure, R1	Structure, R	K_i (nM)	WT IC_{50}/ nM	84V90M IC_{50} / nM	WT	84V90M	30N 82I 88D	48V54V82A	48V54V82S	48V82A90M	46I50V
CO_2H			15	174	3055	>6000	887	ND	ND	ND	ND
$CONH(CH_2)_3PO_3Et_2$		0.009	1.1	12	65	311	74	80	75	74	85
CO_2H			18	299	2344	>6000	3360	ND	ND	ND	ND
$CONH(CH_2)_3PO_3Et_2$		<0.004	2.3	29	176	824	171	233	ND	ND	195
CO_2H		0.091	3.4	27	1548	>6000	>6000	ND	ND	ND	ND
$CONH(CH_2)_3PO_3Et_2$		0.157	1.6	10	188	476	666	240			319



	Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
Structure, R	K_i (nM)	WT IC_{50}/ nM	84V 90M IC_{50} / nM	WT	84V90 M	30N 82I8 8D	48V 54V 82A	48V 54V 82S	48V 82A 90M	46I5 0V
CH_3 (DMP-851)	0.033	3.8	9.4	54	918	69	33	30	22	17
OH	0.65[a]	6.1	77	356	2791	669	294	ND	ND	683
$OCH_2PO_3Et_2$	1.230[a]	23	157	356	>6000	145	175	ND	ND	138
$OCH_2PO_3H_2$	0.809	59	137	1074	>6000	ND	ND	ND	ND	ND
O-mono-Lac-Et	>2.0	93	553	>6000	>6000	ND	ND	ND	ND	ND
O-mono-Lac-Bu	>2.0	25	249	>6000	>6000	ND	ND	ND	ND	ND
CH_2OH	0.017	2.8	31	253	1106	486	413	ND	ND	524
$CH_2OCH_2PO_3Et_2$	2.8	13	123	119	3295	267	430	ND	ND	789
CH_2 $OCH_2PO_3H_2$		42	205	1757	>4243	ND	ND	ND	ND	ND



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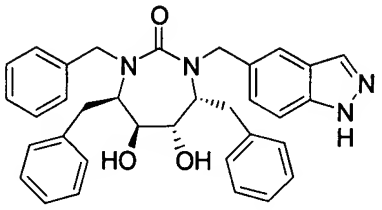

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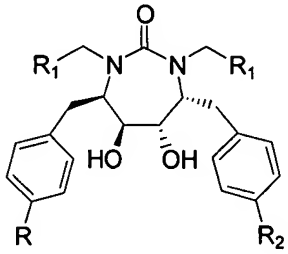
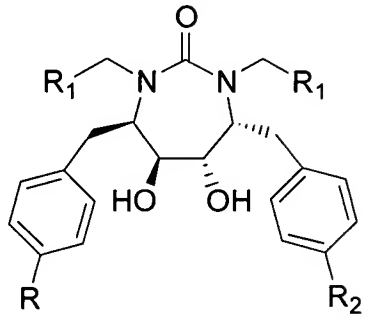
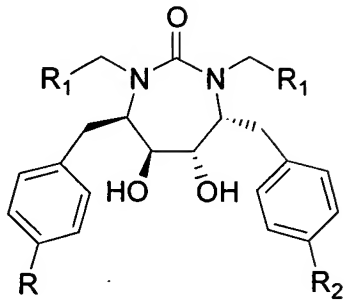


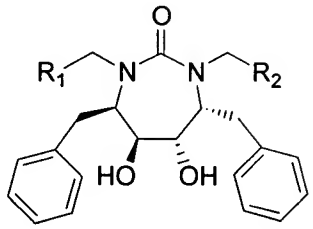
			Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
R	R1	R2	K_i (nM)	WT IC_{50}/ nM	84V90M IC_{50} / nM	WT	84V90M	30N 82I88D	48V54 V82A	48V54 V82S	48V82 A90M	46I50V
---	----	-----	0.033	3.0	9.1	165	819	82	82	73	45	88
---	---	---	0.374	5.8	43.3	193	2312	281	705	ND	ND	772
H	Ph	H		34	631	2492	>6000	3360	ND	ND	ND	ND
OH	Ph	OH		31	397	117	5609	756	2266	ND	ND	928
OH	Ph	$OCH_2PO_3Et_2$		9	40	33	791	92	807	1103	1429	53
H	Ph	$OCH_2PO_3Et_2$	0.656	3.9	48	107	2456	293	1438	1899	3292	589
H	Indazole	H	<0.010	2.5	13	11	22	<8	5.5	8	4	4.0
OH	Indazole	OH	0.0124	0.6	3.5	>6000	2728	7224	ND	ND	ND	ND
OH	Indazole	$OCH_2PO_3Et_2$	0.137	1.1	5.5	1698	1753	1998	ND	ND	ND	ND
H	Indazole	$OCH_2PO_3Et_2$	0.028	1.4	6.2	57	40	68	28	26	32	27

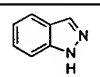
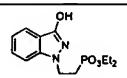
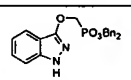
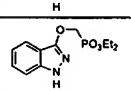
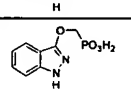
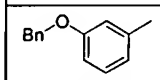
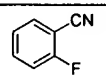
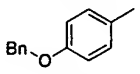
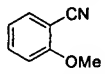
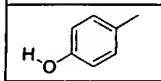
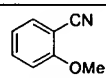


			Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
R	R1	R2	K_i (nM)	WT IC_{50}/ nM	84V90M IC_{50} / nM	WT	84V90M	30N 82I88D	48V54 V82A	48V54 V82S	48V82 A90M	46I50V
---	----	-----	0.033	3.0	9.1	165	819	82	82	73	45	88
OH	Ph	$OCH_2PO_3Et_2$		9	40	33	791	92	807	1103	1429	53
H	Ph	$OCH_2PO_3Et_2$	0.656	3.9	48	107	2456	293	1438	1899	3292	589
OCH_3	Ph	$OCH_2PO_3Et_2$										
OH	Ph-pOH	$OCH_2PO_3Et_2$	<0.01	2.6	18	285	1912	211	986	ND	ND	1107
H	Ph-pOH	$OCH_2PO_3Et_2$	0.319	2.1	33	65	272	90	128	198	126	144
OCH_3	Ph-pOH	$OCH_2PO_3Et_2$	0.045	1.8	17	29	146	23	67	106	48	68
OH	Ph-mNH_2/NHEt	$OCH_2PO_3Et_2$		8.7	67	286	1902	562	789	1781	684	239
H	Ph-mNH_2	$OCH_2PO_3Et_2$	0.126	3.4	39	65	328	16	168	146	74	46
OCH_3	Ph-mNH_2	$OCH_2PO_3Et_2$	<0.01	3.6	56	63	535	18	202	117	102	36
OCH_3	m-pyridine	$OCH_2PO_3Et_2$				115	765	106	1019	970	480	352



			Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
R	R1	R2	K_I (nM)	WT IC_{50}/ nM	84V90M IC_{50} / nM	WT	84V90M	30N 82I88D	48V54 V82A	48V54 V82S	48V82 A90M	46I5 0V
---	----	-----	0.033	3.0	9.1	165	819	82	82	73	45	88
H	Ph-mNH_2	$OCH_2PO_3Et_2$	0.126	3.4	39	65	328	16	168	146	74	46
OCH_3	Ph-mNH_2	$OCH_2PO_3Et_2$	<0.01	3.6	56	63	535	18	202	117	102	36
OCH_3	Ph-mNH_2	$O(CH_2)_2PO_3Et_2$										
OCH_3	Ph-mNH_2	$OCONH(CH_2)_2PO_3Et_2$		11.3	116	74	2265	77	262	214	215	184
OCH_3	Ph-mNH_2	$OCONH(CH_2)PO_3Et_2$		9.9	85	58	2151	68	223	203	185	104
H	Ph-pOH	$OCH_2PO_3Et_2$	0.319	2.1	33	65	272	90	128	222	146	144
OCH_3	Ph-pOH	$OCH_2PO_3Et_2$	0.045	1.8	17	30	148	25	70	129	54	90
OCH_3	Ph-pOH	$OCONH(CH_2)_2PO_3Et_2$		6.6	49	33	495	31	74	51	55	223
---	----	-----	0.033	3.0	9.1	165	819	82	82	73	45	88
H	Ph	$OCH_2PO_3Et_2$	0.656	3.9	48	107	2456	293	1438	1899	3292	589
H	Ph	OH	0.330	15	162	1261	>6000	2952	>6000			
H	Ph	$OCH_2PO_3Bn_2$	0.125	7.4	158	1769	>6000	3135	>6000			
H	Ph	$OCH_2PO_3H_2$	0.386	9.7	210	>6000	>6000	ND	ND			
H	Ph	Mono-lac-Et	0.120	6.6	56	1726	>6000	2793	>6000			
H	Ph	Mono-Ala-Et		5	50	310	2943	238	2851	1948	2450	1250



		Enzymatic assay			Cell-based assay (MT-4) EC_{50} / nM						
R1	R2	K_i (nM)	WT IC_{50}/ nM	84V90M IC_{50} / nM	WT	84V90M	30N 82I88D	48V54V 82A	48V54V 82S	48V82A 90M	46I50V
Phenyl		0.033	3.0	9.1	165	819	82	82	73	45	88
Phenyl		0.423	6.6	85	1226	>6000	869	774	ND	ND	937
Phenyl		0.374	5.8	43.3	193	2312	281	705	ND	ND	772
Phenyl			1095	>2500	>6000	ND	ND	ND	ND	ND	ND
Phenyl											
Phenyl											
Phenyl											
		1.43[a]	302	1142	>6000	>6000	ND	ND	ND	ND	ND
		>5	>2500	ND	5949	ND	ND	ND	ND	ND	ND
		>5	130	3486	2006	3121	ND	ND	ND	ND	ND

All publications and patent applications cited herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although certain embodiments have been described in detail above, those having ordinary skill in the art will clearly understand that many modifications are possible in the embodiments without departing from the teachings thereof. All such modifications are intended to be encompassed within the claims of the invention.

Example: Preliminary Study: Plasma and PBMC Exposure Following Intravenous and Oral Administration of Candidate to Beagle Dogs

The pharmacokinetics of a phosphonate prodrug GS77366 (P1-monoLac-iPr, structure shown below), its active metabolite (metabolite X, or GS77568), and GS8373 were studied in dogs following intravenous and oral administration of the candidate.

Dose Administration and Sample Collection

The in-life phase of this study was conducted in accordance with the USDA Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and followed the standards for animal husbandry and care found in the Guide for the Care and Use of Laboratory Animals, 7th Edition, Revised 1996. All animal housing and study procedures involving live animals were carried out at a facility which had been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care - International (AAALAC).

Each animal in a group of 4 female beagle dogs was given a bolus dose of GS77366 (P1-monoLac-iPr) intravenously at 1 mg/kg in a formulation containing 40% PEG 300, 20% propylene glycol and 40% of 5% dextrose. Another group of 4 female beagle dogs was dosed with GS77366 via oral gavage at 20 mg/kg in a formulation containing 60% Vitamin-E TPGS, 30% PEG 400 and 10% propylene glycol.

Blood samples were collected pre-dose, and at 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr and 24 hr post-dose. Plasma (0.5 to 1 mL) was prepared from each sample and kept at -70°C until analysis. Blood samples (8 mL) were also collected from each dog at 2, 8 and 24 hr post dose in Becton-Dickinson CPT vacutainer tubes. PBMCs were isolated from the blood by centrifugation for 15 minutes at 1500 to 1800 G. After centrifugation, the fraction containing

PBMCs was transferred to a 15 mL conical centrifuge tube and the PBMCs were washed twice with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}. The final wash of the cell pellet was kept at -70°C until analysis.

Measurement of the candidate, metabolite X and GS8373 in plasma and PBMCs

For plasma sample analysis, the samples were processed by a solid phase extraction (SPE) procedure outlined below. Speedisk C18 solid phase extraction cartridges (1 mL, 20 mg, 10 μM, from J.T. Baker) were conditioned with 200 μL of methanol followed by 200 μL of water. An aliquot of 200 μL of plasma sample was applied to each cartridge, followed by two washing steps each with 200 μL of deionized water. The compounds were eluted from the cartridges with a two-step process each with 125 μL of methanol. Each well was added 50 μL of water and mixed. An aliquot of 25 μL of the mixture was injected onto a ThermoFinnigan TSQ Quantum LC/MS/MS system.

The column used in liquid chromatography was HyPURITY® C18 (50 x 2.1 mm, 3.5 μm) from Thermo-Hypersil. Mobile phase A contained 10% acetonitrile in 10 mM ammonium formate, pH 3.0. Mobile phase B contained 90% acetonitrile in 10 mM ammonium formate, pH 4.6. The chromatography was carried out at a flow rate of 250 μL/min under an isocratic condition of 40% mobile phase A and 60% mobile phase B. Selected reaction monitoring (SRM) were used to measure GS77366, GS8373 and Metabolite X with the positive ionization mode on the electrospray probe. The limit of quantitation (LOQ) was 1 nM for GS77366, GS8373 and GS77568 (Metabolite X) in plasma.

For PBMC sample analysis, phosphate buffered saline (PBS) was added to each PBMC pellet to bring the total sample volume to 500 μL in each sample. An aliquot of 150 μL from each PBMC sample was mixed with an equal volume of methanol, followed by the addition of 700 μL of 1% formic acid in water. The resulting mixture was applied to a Speedisk C18 solid phase extraction cartridge (1 mL, 20 mg, 10 um, from J.T. Baker) which had been conditioned as described above. The compounds were eluted with methanol after washing the cartridge 3 times with 10% methanol. The solvent was evaporated under a stream of N_2, and the sample was reconstituted in 150 μL of 30% methanol. An aliquot of 75 μL of the solution was injected for LC/MS/MS analysis. The limit of quantitation was 0.1 ng/mL in the PBMC suspension.

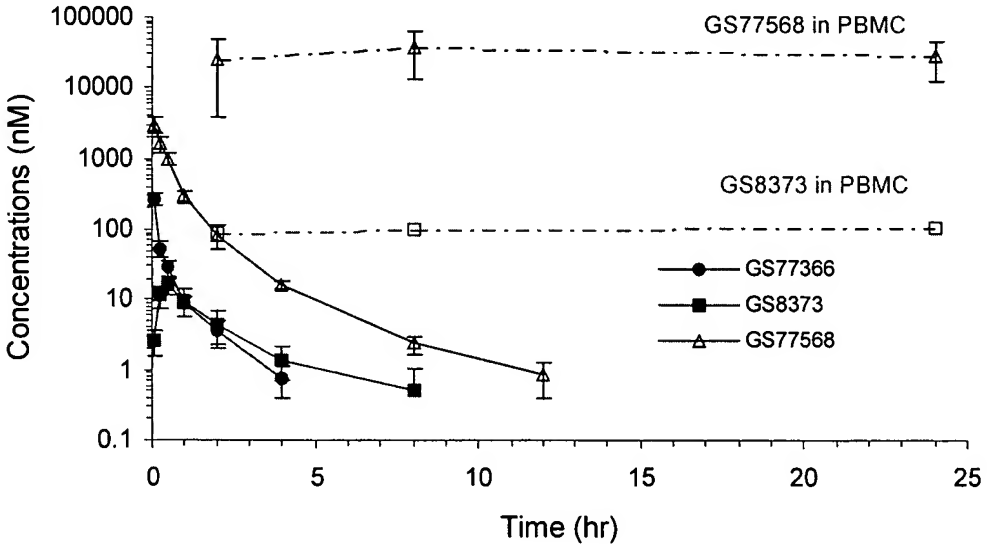
Pharmacokinetic Calculations

The pharmacokinetic parameters were calculated using WinNonlin. Noncompartmental analysis was used for all pharmacokinetic calculation. The intracellular concentrations in PBMCs were calculated from the measured concentrations in PBMC suspension on the basis of a reported volume of 0.2 picoliter/cell (B.L. Robins, R.V. Srinivas, C. Kim, N. Bischofberger, and A. Fridland, (1998) *Antimicrob. Agents Chemother.* 42, 612).

Plasma and PBMC Concentration-time Profiles

The following shows the concentration-time profiles of GS77366, GS77568 and GS8373 in plasma and PBMCs following intravenous dosing of GS77366 at 1 mg/kg in dogs. The data demonstrate that the prodrug can effectively deliver the active components (metabolite X and GS8373) into cells that are primarily responsible for HIV replication, and that the active components in these cells had much longer half-life than in plasma.

Pharmacokinetic profiles of GS77366, GS77568 and GS8373 in plasma and PBMCs following intravenous administration of GS77366 at 1 mg/kg in dogs



The pharmacokinetic properties of GS77568 in PBMCs following oral administration of GS77366 in dogs are compared with that of nelfinavir and amprenavir, two marketed HIV protease inhibitors. These data show that the active component (GS77568) from the phosphonate prodrug had sustained levels in PBMCs compared to nelfinavir and amprenavir.

Concentration-time profiles of GS77568, nelfinavir and amprenavir in PBMCs following oral administration of GS77366 (20 mg/kg), nelfinavir (17.5 mg/kg) and amprenavir (20 mg/kg) in dogs

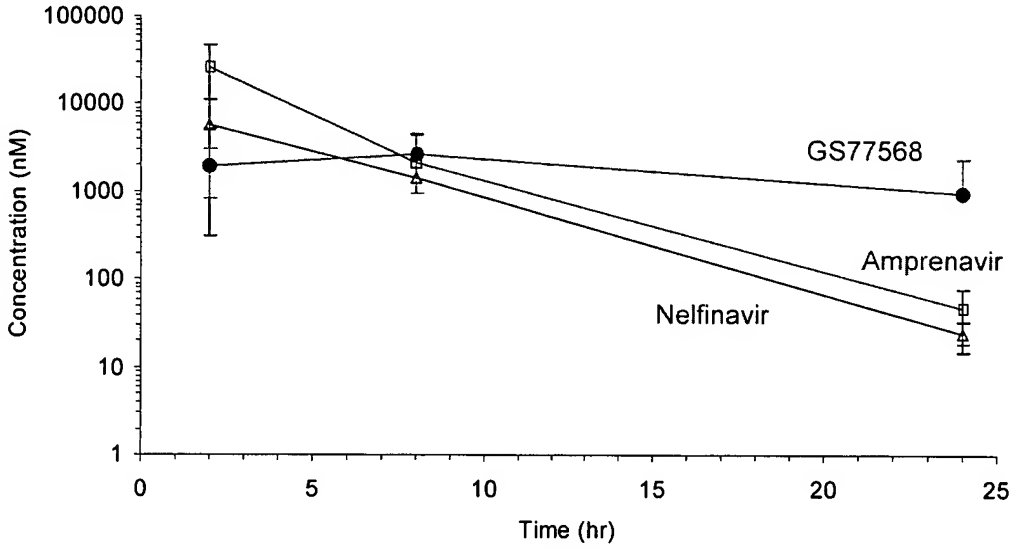


Table 1a: Comparison of GS77568 with nelfinavir and amprenavir in PBMCs following oral administration in beagle dogs

Compound	Dose	$t_{1/2}$ (hr)	$AUC_{(2\text{-}24\ hr)}$
Nelfinavir	17.5 mg/kg	3.0 hr	33,000 nM·hr
Amprenavir	20 mg/kg	1.7 hr	102,000 nM·hr
GS77568	20 mg/kg of GS77366	> 20 hr	42,200 nM·hr

Intracellular Metabolism/*In Vitro* Stability

1. Uptake and Persistence in MT2 cells, quiescent and stimulated PBMC

The protease inhibitor (PI) phosphonate prodrugs undergo rapid cell uptake and metabolism to produce acid metabolites including the parent phosphonic acid. Due to the presence of charges, the acid metabolites are significantly more persistent in the cells than non-charged PI's. In order to estimate the relative intracellular levels of the different PI prodrugs, three compounds representative of three classes of phosphonate PI prodrugs – bisamidate

phosphonate, monoamidate phenoxy phosphonate and monolactate phenoxy phosphonate (Figure 1) were incubated at 10 μM for 1 hr with MT-2 cells, stimulated and quiescent peripheral blood mononuclear cells (PBMC) (pulse phase). After incubation, the cells were washed, resuspended in the cell culture media and incubated for 24 hr (chase phase). At specific time points, the cells were washed, lysed and the lysates were analyzed by HPLC with UV detection. Typically, the cell lysates were centrifuged and 100 uL of the supernatant were mixed with 200 μL of 7.5 uM amprenavir (Internal Standard) in 80% acetonitrile/20% water and injected into an HPLC system (70 μL).

HPLC Conditions:

Analytical Column: Prodigy ODS-3, 75 x 4.6, 3u + C18 guard at 40°C

Gradient:

Mobile Phase A: 20 mM ammonium acetate in 10% ACN/90% H_2O

Mobile Phase B: 20 mM ammonium acetate in 70% ACN/30% H_2O

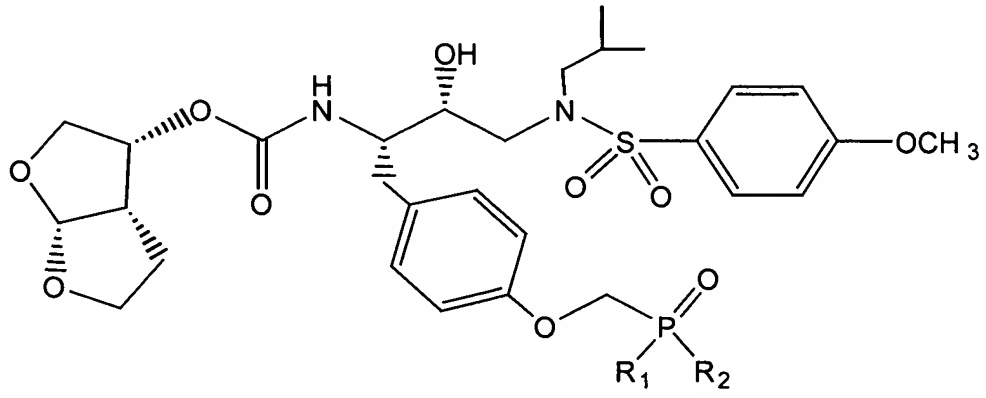
30-100%B in 4 min, 100%B for 2 min, 30%B for 2 min at 2.5 mL/min.

Run Time: 8 min

UV Detection @ 245 nm

Concentrations of Intracellular metabolites were calculated based on cell volume 0.2 μL/mln cells for PBMC and 0.338 μL / mln (0.676 uL / mL) for MT-2 cells.

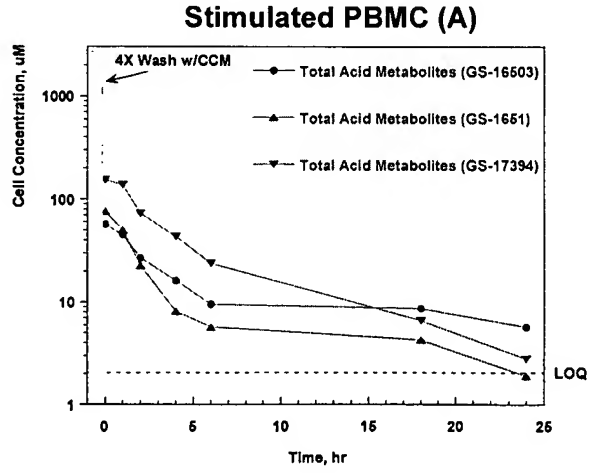
Chemical Structures of Selected Protease Inhibitor Phosphonate Prodrugs and Intracellular Metabolites

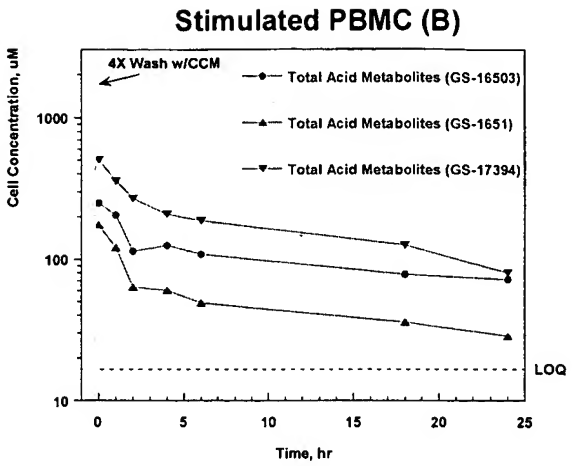


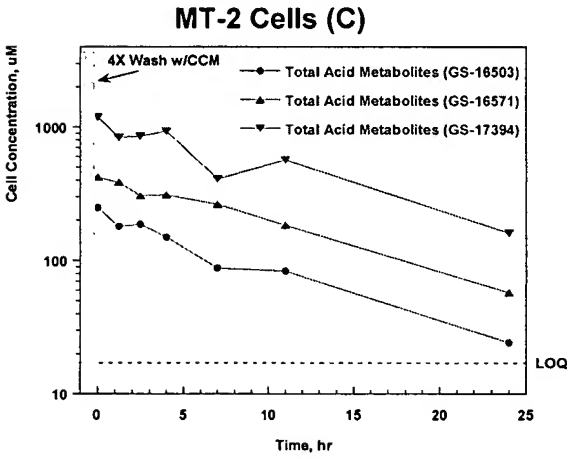
GS No.	R1	R2	EC_{50} (nM)
8373	OH	OH	4,800±1,800
16503	$HNCH(CH_3)COOBu$	$HNCH(CH_3)COOBu$	6.0±1.4
16571	OPh	$HNCH(CH_3)COOEt$	15±5
17394	OPh	$OCH(CH_3)COOEt$	20±7
16576	OPh	$HNCH(CH_2CH_3)COOEt$	12.6±4.8
Met X	OH	$HNCH(CH_3)COOH$	>10,000
Met LX	OH	$OCH(CH_3)COOEt$	1750±354

The foregoing data demonstrates that there was a significant uptake and conversion of all 3 compounds in all cell types. The uptake in the quiescent PBMC was 2-3-fold greater than in the stimulated cells. GS-16503 and GS-16571 were metabolized to Metabolite X and GS-8373. GS-17394 metabolized to the Metabolite LX. Apparent intracellular half-lives were similar for all metabolites in all cell types (7-12 hr).

Persistence of Total Acid Metabolites of Protease Inhibitor Prodrugs in Stimulated (A), Quiescent PBMC (B) and MT-2 Cells (C) (1 hr, 10 uM Pulse, 24 hr Chase)







2. Uptake and Persistence in Stimulated and Quiescent T-cells

Since HIV mainly targets T-lymphocytes, it is important to establish the uptake , metabolism and persistence of the metabolites in the human T-cells. In order to estimate the relative intracellular levels of the different PI prodrugs, GS-16503, 16571 and 17394 were incubated at 10 μM for 1 hr with quiescent and stimulated T-cells (pulse phase). The prodrugs were compared with a non-prodrug PI, nelfinavir. After incubation, the cells were washed, resuspended in the cell culture media and incubated for 4 hr (chase phase). At specific time points, the cells were washed, lysed and the lysates were analyzed by HPLC with UV detection. The sample preparation and analysis were similar to the ones described for MT-2 cells, quiescent and stimulated PBMC.

Table 1b demonstrates the levels of total acid metabolites and corresponding prodrugs in T-cells following pulse/chase and continuous incubation. There was significant cell uptake/metabolism in T-lymphocytes. There was no apparent difference in uptake between stimulated and quiescent T-lymphocytes. There was significantly higher uptake of phosphonate PI's than nelfinavir. GS17394 demonstrates higher intracellular levels than GS16571 and GS16503. The degree of conversion to acid metabolites varied between different prodrugs. GS-17394 demonstrated the highest degree of conversion, followed by GS-16503 and GS-16571. The metabolites, generally, were an equal mixture of the mono-phosphonic acid metabolite and GS-8373 except for GS-17394, where Metabolite LX was stable, with no GS-8373 formed.

Table 1b: Intracellular Levels of Metabolites and Intact Prodrug Following Continuous and 1 hr Pulse/4 hr Chase Incubation (10 μM/0.7 mln cells/1 mL) of 10 μM PI Prodrugs and Nelfinavir with Quiescent and Stimulated T-cells

		Continuous Incubation				1 hr Pulse /4 hr Chase			
		Quiescent T-cells		Stimulated T-cells		Quiescent T-cells		Stimulated T-cells	
Compound	Time (h)	Acid Met (μM)	Prodrug (μM)	Acid Met (μM)	Prodrug (μM)	Acid Met (μM)	Prodrug (μM)	Acid Met (μM)	Prodrug (μM)
16503	0	1180	42	2278	0	2989	40	1323	139
	2	3170	88	1083	116	1867	4	1137	31
	4	5262	0	3198	31	1054	119	1008	0
16571	0	388	1392	187	1417	1042	181	858	218
	2	947	841	1895	807	1170	82	1006	35
	4	3518	464	6147	474	1176	37	616	25
17394	0	948	1155	186	1194	4480	14	2818	10
	2	7231	413	3748	471	2898	33	1083	51
	4	10153	167	3867	228	1548	39	943	104
Nelfinavir	0		101		86		886		1239
	2		856		846		725		770
	4		992		1526		171		544

3. PBMC Uptake and Metabolism of Selected PI Prodrugs Following 1-hr Incubation in MT-2 Cells at 10, 5 and 1 μM

To determine if the cell uptake/metabolism is concentration dependent, selected PI's were incubated with the 1 mL of MT-2 cell suspension (2.74 mln cells/mL) for 1 hr at 37°C at 3

different concentrations: 10, 5 and 1 μM. Following incubation, cells were washed twice with the cell culture medium, lysed and assayed using HPLC with UV detection. The sample preparation and analysis were similar to the ones described for MT-2 cells, quiescent and stimulated PBMC. Intracellular concentrations were calculated based on cell count, a published single cell volume of 0.338 pl for MT-2 cells, and concentrations of analytes in cell lysates. Data are shown in Table 2a.

Uptake of all three selected PI's in MT-2 cells appears to be concentration-independent in the 1-10 uM range. Metabolism (conversion to acid metabolites) appeared to be concentration-dependent for GS-16503 and GS-16577 (3-fold increase at 1 uM vs. 10 uM) but independent for GS-17394 (monolactate). Conversion from a respective metabolite X to GS-8373 was concentration-independent for both GS-16503 and GS-16577 (no conversion was observed for metabolite LX of GS-17394).

Table 2a: Uptake and Metabolism of Selected PI Prodrugs Following 1-hr Incubation in MT-2 Cells at 10, 5 and 1 μM

Compound	Extracellular Concentration, μM	Cell-Assosiated Prodrug and Metabolites Concentration, μM				% Conversion to acid metabolites
		Metabolite X	GS8373	Prodrug	Total	
GS-17394	10	1358	0	635	1993	68
	5	916	0	449	1365	67
	1	196	0	63	260	76
GS-16576	10	478	238	2519	3235	22
	5	250	148	621	1043	40
	1	65	36	61	168	64
GS-16503	10	120	86	1506	1712	12
	5	58	60	579	697	17
	1	12	18	74	104	29

* For GS16576, Metabolite X is mono-aminobutyric acid

4. PBMC Uptake and Metabolism of Selected PI Candidates Following 1-hr Incubation in Human Whole Blood at 10 uM

In order to estimate the relative intracellular levels of the different PI prodrugs candidates under conditions simulating the in vivo environment, compounds representative of three classes of phosphonate PI prodrugs – bisamidate phosphonate (GS-16503), monoamidate phenoxy phosphonate (GS-16571) and monolactate phenoxy phosphonate(GS-17394) (Figure 1) were incubated at 10 μM for 1 hr with intact human whole blood at 37°C. After incubation, PBMC were isolated, then lysed and the lysates were analyzed by HPLC with UV detection.

The results of analysis are shown in Table 3. There was significant cell uptake/metabolism following incubation in whole blood. There was no apparent difference in uptake between GS-16503 and GS-16571. GS-17394 demonstrated significantly higher intracellular levels than GS-16571 and GS-16503.

The degree of conversion to acid metabolites varies between different prodrugs after 1 hr incubation. GS-17394 demonstrated the highest degree of conversion, followed by GS-16503 and GS-16571. The metabolites, generally, were an equimolar mixture of the mono-phosphonic acid metabolite and GS-8373 (parent acid) except for GS-17394, where Metabolite LX was stable with no GS-8373 formed.

Table 3a: PBMC Uptake and Metabolism of Selected PI Prodrugs Following 1-hr Incubation in Human Whole Blood at 10 uM (Mean ± SD, N=3)

GS#	Intracellular Prodrug and Metabolites Concentration, uM			Major Intracellular Metabolites
	Acid Metabolite	Prodrug, μM	Total, μM	
16503	279 ± 47	61 ± 40	340 ± 35	X , GS-8373
16571	319 ± 112	137 ± 62	432 ± 208	X, GS-8373
17394	629 ± 303	69 ± 85	698 ± 301	LX

* PBMC Intracellular Volume = 0.2 μL/mln

5. Distribution of PI Prodrug Candidates in PBMC

In order to compare distribution and persistence of PI phosphonate prodrugs with those of non-prodrug PI's, GS-16503, GS-17394 and nelfinavir, were incubated at 10 μM for 1 hr with PBMC (pulse phase). After incubation, the cells were washed, resuspended in the cell culture media and incubated for 20 more hr (chase phase). At specific time points, the cells were washed and lysed. The cell cytosol was separated from membranes by centrifugation at 9000 x g. Both cytosol and membranes were extracted with acetonitrile and analyzed by HPLC with UV detection.

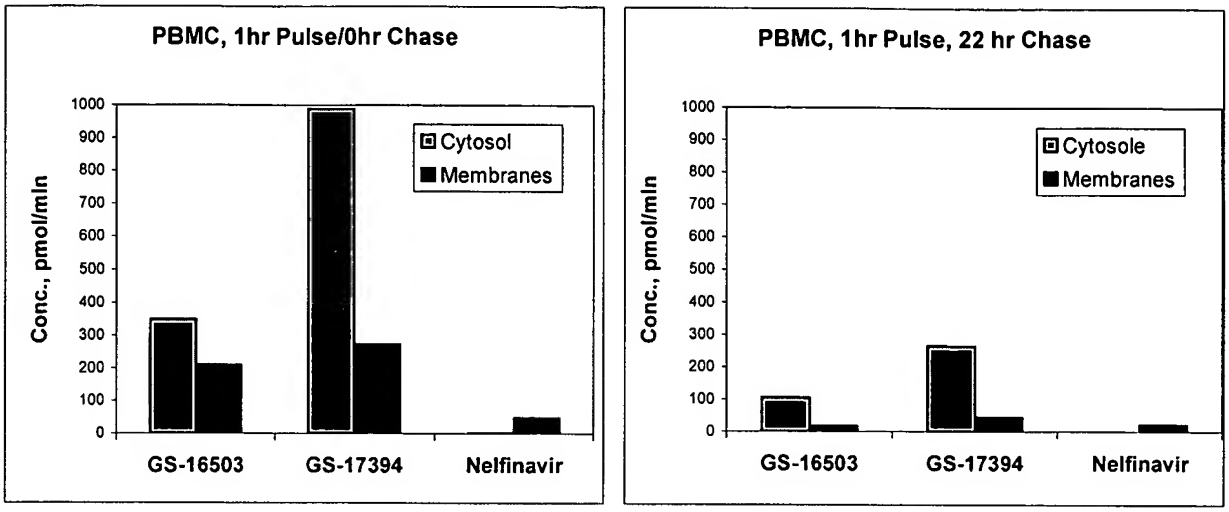
Table 4a and the accompanying bar graphs below show the levels of total acid metabolites and corresponding prodrugs in the cytosol and membranes before and after the 22 hr chase. Both prodrugs exhibited complete conversion to the acid metabolites (GS-8373 and X for GS-16503 and LX for GS-17394, respectively). The levels of the acid metabolites of the PI phosphonate prodrugs in the cytosol fraction were 2-3-fold greater than those in the membrane fraction after the 1 hr pulse and 10-fold greater after the 22 hr chase. Nelfinavir was present only in the membrane fractions. The uptake of GS-17394 was about 3-fold greater than that of GS-16503 and 30-fold greater than nelfinavir.

The metabolites were an equimolar mixture of metabolite X and GS-8373 (parent acid) for GS-16503 and only metabolite LX for GS-17394.

Table 4a: Uptake and Cell Distribution of Metabolites and Intact Prodrugs Following Continuous and 1 hr Pulse/22 hr Chase Incubation of 10 uM PI Prodrugs and Nelfinavir with Quiescent PBMC

GS#	Cell Type	Fraction	Cell-Associated PI, pmol/mln cells			
			1 hr Pulse/ 0 hr Chase		1 hr Pulse/ 22 hr Chase	
			Acid Metabolites	Prodrug	Acid Metabolites	Prodrug
GS-16503	PBMC	Membrane	228	0	9	0
GS-16503	PBMC	Cytosol	390	0	130	0
GS-17394	PBMC	Membrane	335	0	26	0
GS-17394	PBMC	Cytosol	894	0	249	0
Nelfinavir	PBMC	Membrane		42		25
Nelfinavir	PBMC	Cytosol		0		0

Uptake and Cell Distribution of Metabolites and Intact Prodrugs Following 1 hr Pulse/22 hr Chase Incubation of 10 uM PI Prodrugs and Nelfinavir with Quiescent PBMC



6. PBMC Extract/Dog Plasma/Human Serum Stability of Selected PI Prodrug Candidates

The *in vitro* metabolism and stability of the PI phosphonate prodrugs were determined in PBMC extract, dog plasma and human serum. Biological samples listed below (120 μL) were transferred into an 8-tube strip placed in the aluminum 37°C heating block/holder and incubated at 37°C for 5 min. Aliquots (2.5 μL) of solution containing 1 mM of test compounds in DMSO, were transferred to a clean 8-tube strip, placed in the aluminum 37°C heating block/holder. 60 μL aliquots of 80% acetonitrile/20% water containing 7.5 μM of amprenavir as an internal standard for HPLC analysis were placed into five 8-tube strips and kept on ice/refrigerated prior to use. An enzymatic reaction was started by adding 120 μL aliquots of a biological sample to the strip with the test compounds using a multichannel pipet. The strip was immediately vortex-mixed and the reaction mixture (20 μL) was sampled and transferred to the Internal Standard/ACN strip. The sample was considered the time-zero sample (actual time was 1-2 min). Then, at specific time points, the reaction mixture (20 μL) was sampled and transferred to the corresponding IS/ACN strip. Typical sampling times were 6, 20, 60 and 120 min. When all time points were sampled, an 80 μL aliquot of water was added to each tube and strips were centrifuged for 30 min at 3000xG. The supernatants were analyzed with HPLC under the following conditions:

Column: Inertsil ODS-3, 75 x 4.6 mm, 3 μm at 40°C.

Mobile Phase A: 20 mM ammonium acetate in 10%ACN/90%water

Mobile Phase B 20 mM ammonium acetate in 70%ACN/30%water

Gradient: 20% B to 100% B in 4 min, 2 min 100% B, 2 min 20% B

Flow Rate: 2 mL/min

Detection: UV at 243 nm

Run Time: 8 min

The biological samples evaluated were as follows:

PBMC cell extract was prepared from fresh cells using a modified published procedure (A. Pompon, I. Lefebvre, J.-L. Imbach, S. Kahn, and D. Farquhar, *Antiviral Chemistry & Chemotherapy*, 5, 91 - 98 (1994)). Briefly, the extract was prepared as following: The cells were separated from their culture medium by centrifugation (1000 g, 15 min, ambient temperature). The residue (about 100 μL, 3.5 x 10^8 cells) was resuspended in 4 mL of a buffer (0.010 M HEPES, pH 7.4, 50 mM potassium chloride, 5 mM magnesium chloride and 5 mM dl-dithiothreitol) and sonicated. The lysate was centrifuged (9000 g, 10 min, 4°C) to remove membranes. The upper layer (0.5 mg protein/mL) was stored at –70°C. The reaction mixture contained the cell extract at about 0.5 mg protein/mL.

Human serum (pooled normal human serum from George King Biomedical Systems, Inc.). Protein concentration in the reaction mixture was about 60 mg protein/mL.

Dog Plasma (pooled normal dog plasma (EDTA) from Pel Freez, Inc.). Protein concentration in the reaction mixture was about 60 mg protein/mL.

Table 5a: PBMC Extract/Dog Plasma/Human Serum Stability of Selected PI Prodrugs

GS#	PBMC Extract[1] $T_{1/2}$, min	Dog Plasma $T_{1/2}$, min	Human Serum $T_{1/2}$, min	HIV EC_{50} (nM)
16503	2	368	>>400	6.0 ± 1.4
16571	49	126	110	15 ± 5
17394	15	144	49	20 ± 7

Example: Pharmacokinetics in Plasma and PBMC Following Intravenous or Oral Administration of Candidate compounds to Beagle Dogs; Method for Determining Intracellular Residence Time

The pharmacokinetics of several candidate compounds and their active metabolites were studied in beagle dogs following intravenous or oral administration of each candidate compound.

Dose Administration and Sample Collection

Each dosing group consisted of 3 male beagle dogs that were fasted overnight before dosing. For intravenous administration, each dog was dosed with the candidate compound at 1 mg/kg via the cephalic vein as a slow bolus injection over approximately 1 minute. Blood samples (1-2 mL) were collected from the jugular vein pre-dose, and at 2 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr and 24 hr post-dose into tubes containing EDTA as the anticoagulant. For oral administration, each dog was dosed with the candidate compound at 4 mg/kg through oral gavage. Blood samples (1-2 mL) were collected pre-dose, and at 5 min, 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr and 12 hr post-dose into tubes containing EDTA as the anticoagulant. The blood samples were stored on ice and plasma samples were obtained by centrifugation within 1 hour after blood collection. The plasma samples were stored at approximately -70°C until analysis for the concentrations of the candidate compound and its metabolites in plasma.

Another set of blood samples was also collected from the jugular vein for evaluation of the concentrations of candidate compound and its metabolites in peripheral blood mononuclear cells (PBMCs). Approximately 8 mL of blood was collected either at 1 hr, 4 hr, 8 hr and 24 hr post-dose or at 2 hr, 8 hr and 24 hr post-dose from the jugular vein into tubes containing EDTA as the anticoagulant. An equal volume of sterile phosphate buffered saline (PBS) was mixed with each blood sample. The mixture was laid over 15 mL of Ficoll-Paque (Amersham Biosciences) in a 50 mL conical tube. The tube was centrifuged at approximately 500 g for 30 min at room temperature. The upper layer containing plasma was drawn off and discarded. The layer below the plasma layer is enriched with PBMCs. This layer was collected with a clean pipette and transferred to a 15 mL conical tube. The PBMC suspension was centrifuged at approximately 500 g for 10 min at room temperature. The resulting pellet was resuspended in 5 mL of sterile PBS and then centrifuged at approximately 500 g for 10 min at room temperature. The supernatant was removed and 0.5 mL of acetonitrile was added to the pellet. The tube was

vortexed, sealed and stored at -70°C until analysis for concentrations of the candidate compound and its metabolites.

Determination of the concentrations of the candidate compound and its metabolites in plasma

The plasma concentrations of the candidate compound and its metabolites were determined by an LC/MS/MS assay. The plasma samples were processed with a solid phase extraction (SPE) procedure outlined below. Speedisk C18 solid phase extraction cartridges (1 mL, 20 mg, 10 um, from J.T. Baker) in a 96-well plate were conditioned with 200 uL of methanol followed by 200 uL of water. An aliquot of 200 uL of plasma sample was applied to each cartridge, followed by two washing steps each with 200 uL of deionized water. The analytes were eluted from the cartridges by a two-step process each with 125 uL of methanol. Each well was added 50 uL of water and mixed to reduce the organic strength. An aliquot of 25 uL of the mixture was injected onto a ThermoFinnigan TSQ Quantum LC/MS/MS system.

The column used in liquid chromatography (LC) was HyPURITY® C18 (50 x 2.1 mm, 3.5 um) from Thermo-Hypersil. Mobile phase A contained 10% acetonitrile in 10 mM ammonium formate, 0.1% formic acid. Mobile phase B contained 90% acetonitrile in 10 mM ammonium formate, 0.1% formic acid. The chromatography was carried out at a flow rate of 250 µL/min under an isocratic condition of 40% mobile phase A and 60% mobile phase B. Selected reaction monitoring (SRM) were used to measure the candidate compound and its metabolites simultaneously with the positive ionization mode on the electrospray probe. The limit of quantitation (LOQ) was 1 nM for the candidate compound and its metabolites in plasma.

Determination of the concentrations of the candidate compound and its metabolites in PBMCs

The concentrations of the candidate compound and its metabolites in PBMCs were determined by an LC/MS/MS assay. The PBMC samples were filtered through a CAPTIVA™ filtration plate with 0.2 µm pore size. An aliquot of 250 µL of the filtrate was evaporated under a stream of nitrogen. The samples were reconstituted in 75 µL of 20% acetonitrile in 0.1% formic acid. An aliquot of 25 uL of the solution was injected onto a ThermoFinnigan TSQ Quantum LC/MS/MS system.

The column used in liquid chromatography was HyPURITY® C18 (50 x 2.1 mm, 3.5 um) from Thermo-Hypersil. Mobile phase A (MPA) contained 10% acetonitrile in 10 mM ammonium formate, 0.1% formic acid. Mobile phase B (MPB) contained 90% acetonitrile in 10

mM ammonium formate, 0.1% formic acid. The chromatography was carried out at a flow rate of 300 μL/min with a gradient elution program: 5% MPB from 0 to 1.5 min; 5-95% MPB from 1.5 to 1.6 min; 95% MPB from 1.6 to 3.5 min; 95-5% MPB from 3.5 to 3.6 min; 5% MPB till the end of the program (6 min). The first 2 min of the LC flow was diverted to waste to alleviate salt buildup in the probe of the mass spectrometer. Selected reaction monitoring was used to measure the candidate compound and its metabolites simultaneously with the positive ionization mode on the electrospray probe. The limit of quantitation (LOQ) was 0.1 nM for the candidate compound and its metabolites in PBMC suspension.

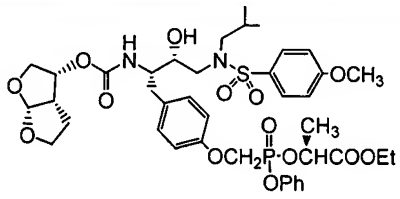
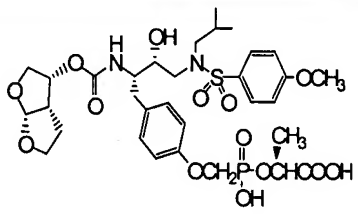
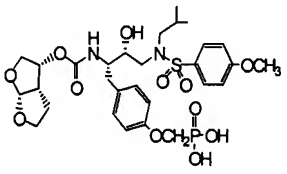
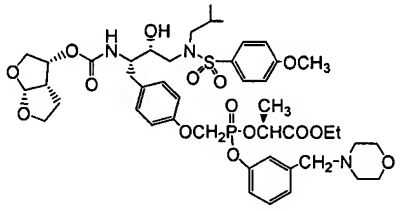
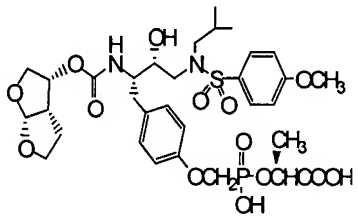
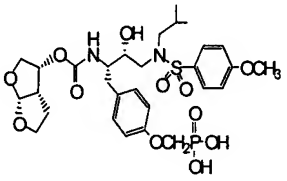
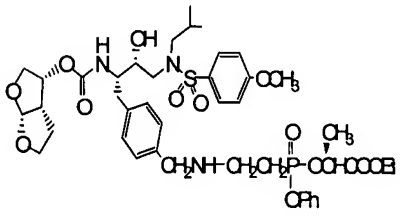
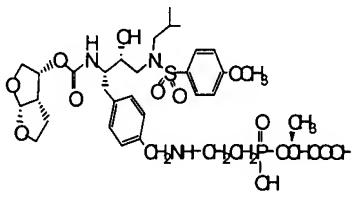
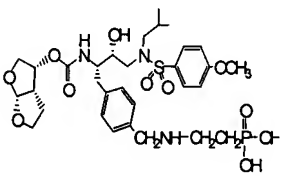
Pharmacokinetic Calculations

The pharmacokinetic parameters were calculated using WinNonlin. Noncompartmental analysis was used for all pharmacokinetic calculation. The intracellular concentrations in PBMCs were extrapolated from the measured concentrations in PBMC suspension on the basis of a reported volume of 0.2 picoliter/cell (B.L. Robins, R.V. Srinivas, C. Kim, N. Bischofberger, and A. Fridland, (1998) *Antimicrob. Agents Chemother.* 42, 612).

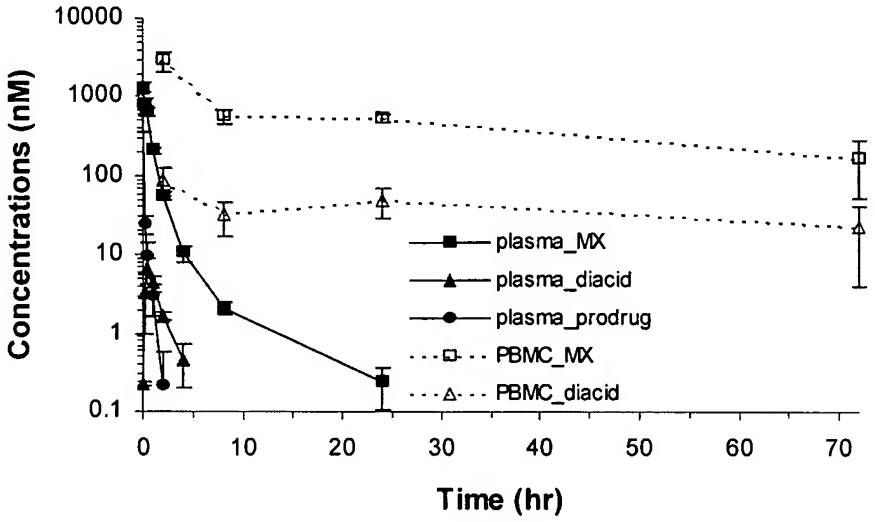
Pharmacokinetic Profiles in Plasma and PBMC

Shown below are the concentration-time profiles of three phosphonate candidate compounds (GS-1, GS-2 and GS-3) and their metabolites in plasma and PBMCs following intravenous administration of each candidate compound at 1 mg/kg in dogs. The last profile shows the concentration-time profiles of GS-3 and its metabolites in plasma and PBMC following oral administration of GS-3 at 4 mg/kg in dogs. The chemical structures of the candidate compounds and their metabolites are shown in Table 1aa. The data demonstrate that the candidate compounds can effectively deliver the active components (metabolite X and diacid) into cells that are primarily associated with HIV activity, and that the half-lives of the active components in these cells are much longer than in plasma.

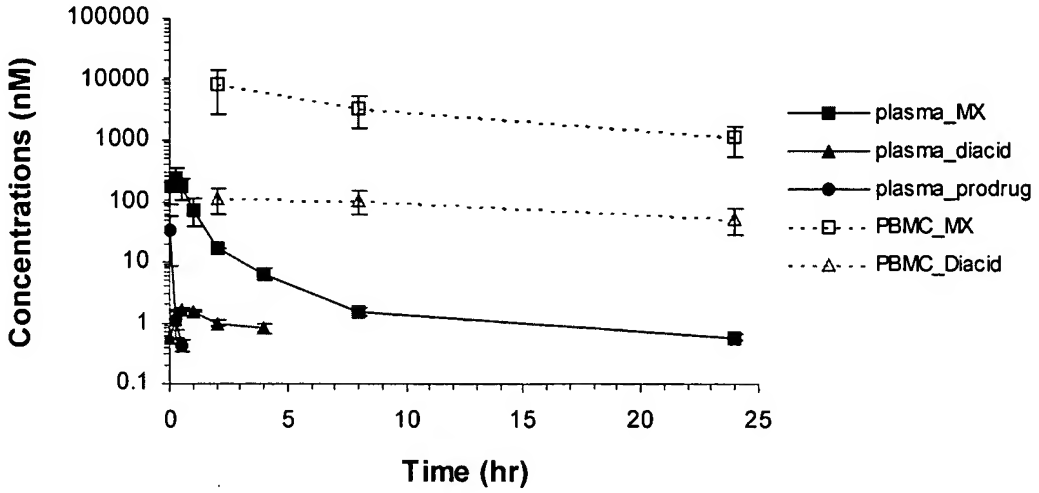
Table 1aa: Chemical Structures of Candidate compounds and Their Metabolites.

	Candidate compound	Metabolites	
		Metabolite X (MX)	Diacid
GS-1			
GS-2			
GS-3			

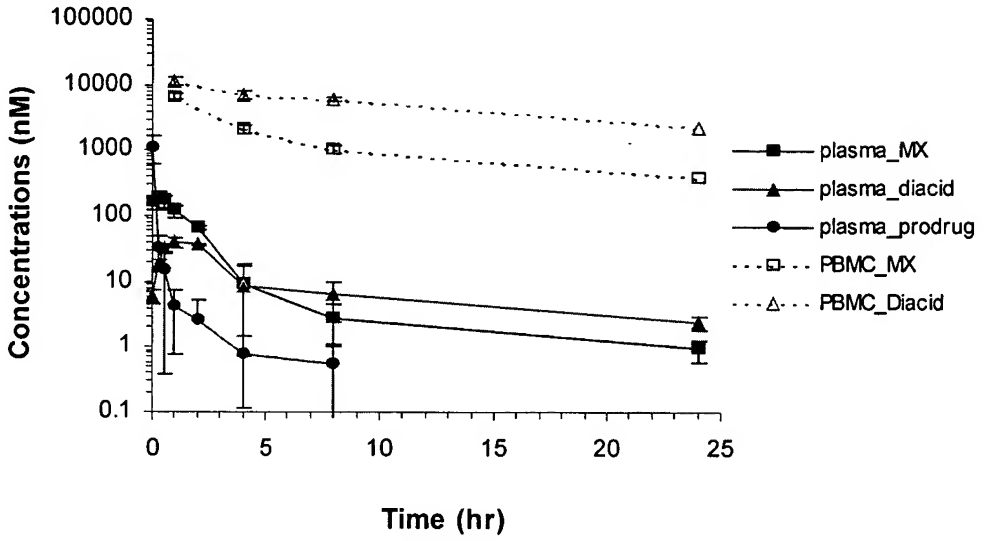
Pharmacokinetic profiles of GS-1 and its metabolites in plasma and PBMCs following intravenous administration of GS-1 at 1 mg/kg in dogs



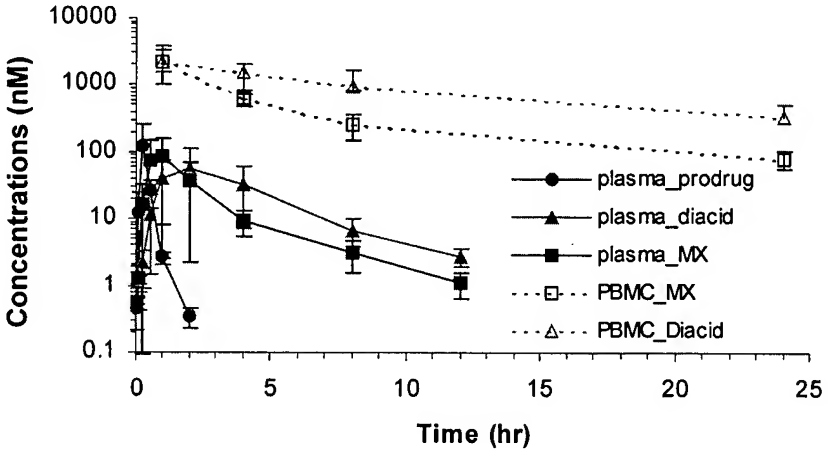
Pharmacokinetic profiles of GS-2 and its metabolites in plasma and PBMCs following intravenous administration of GS-2 at 1 mg/kg in dogs



Pharmacokinetic profiles of GS-3 and its metabolites in plasma and PBMCs following intravenous administration of GS-3 at 1 mg/kg in dogs

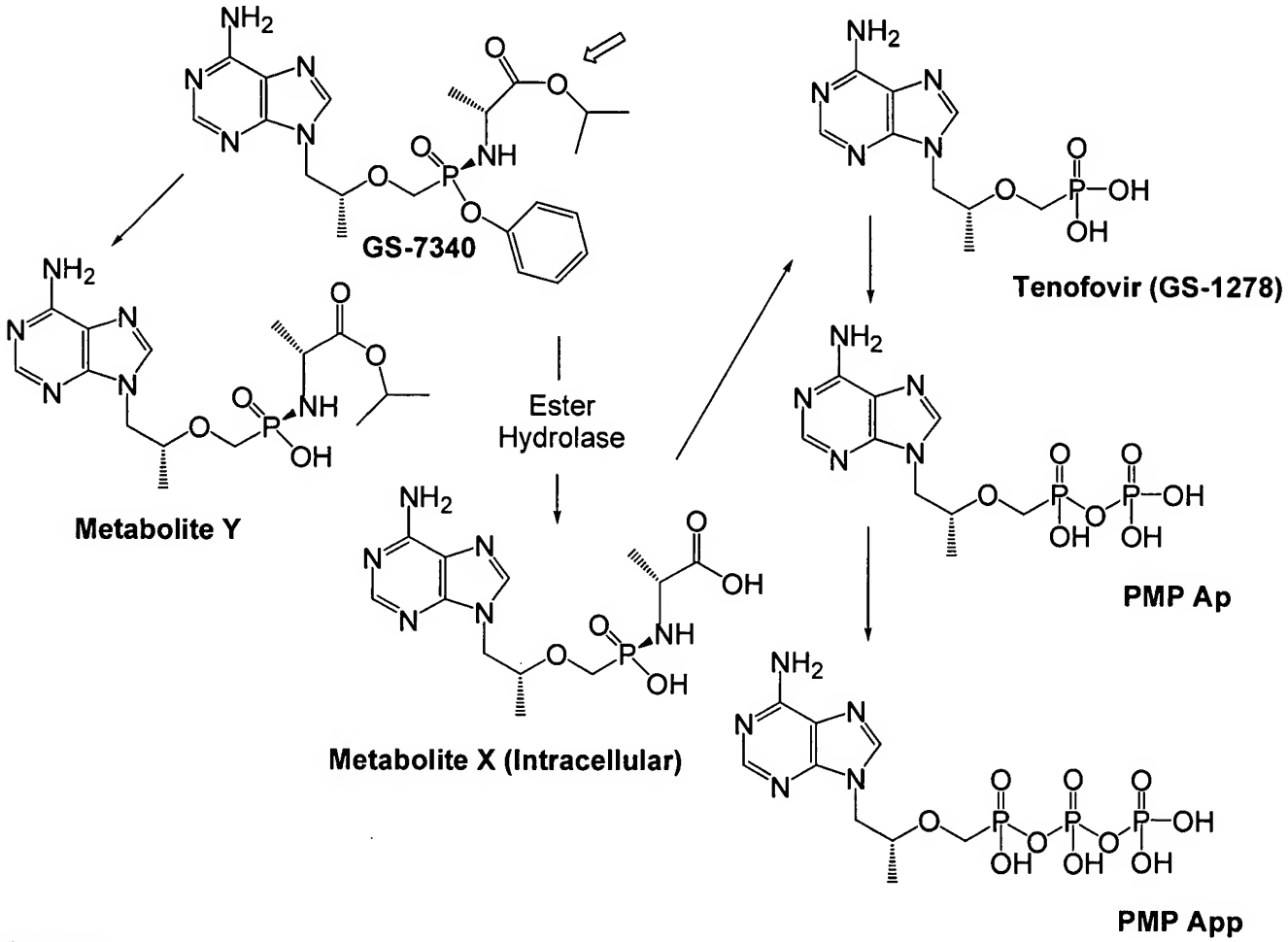


Pharmacokinetic profiles of GS-3 and its metabolites in plasma and PBMCs following oral administration of GS-3 at 4 mg/kg in dogs



Example: Purification and Biochemical Characterization of GS-7340 Ester Hydrolase

Major Metabolites of GS-7340



Scheme 1

Metabolism of GS-7340

There is broad consensus that the bioactivation of nucleotide amidate triesters follows a general scheme (Scheme 1) (Valette, 1996; McGuigan, 1998a, 1998b; Saboulard, 1999; Siddiqui, 1999). Step A is the hydrolysis of the amino acid carboxylic ester. A nucleophilic attack by the carboxylic acid of the phosphorous (Step B) is believed to initiate the formation of the 5-membered cyclic intermediate which in turn is quickly hydrolyzed to the monoamidate diester (referred to as the amino acid nucleoside monophosphate, AAM, or metabolite X, Step C). This

compound is considered an intracellular depot form of the antiviral nucleoside. Various enzymes as well as non-enzymatic catalysis have been implicated in Step D which is the hydrolysis of the amide bond resulting in the formation of the nucleotide. The nucleotide is activated by enzymatic phosphorylation to nucleotide di- and tri-phosphates.

In the case of GS-7340, the efficient conversion of this pro-drug to the amino acid nucleoside monophosphate (Metabolite X) is a necessary step for the observed accumulation of Metabolite X is peripheral blood mononuclear cells (PBMC). Purification of the Enzyme(s) responsible for the cleavage of GS-7340 amino acid carboxylic ester resulting in the formation of Metabolite X is the subject of this example.

Ester Hydrolase Assay

The enzymatic production of metabolite X from GS-7340 was monitored using the following Ester Hydrolase assay: Varying amounts of peripheral blood mononuclear cell (PBMC) extracts, column fractions or pools were incubated with [^{14}C] GS-7340 at 37°C for 10 – 90 min. The production of [^{14}C] Metabolite X was monitored by measuring the amount of radioactivity retained on an anion exchange resin (DE-81). HPLC and mass spectrometry analysis of the reaction mixture and radioactivity retained on the filter confirmed that only [^{14}C]-Metabolite X bound the DE-81 filter. Under the assay conditions, the more hydrophobic [^{14}C] GS-7340 is not retained on the DE-81 membrane. The final reaction conditions were: 25 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.5, 100mM NaCl, 1 mM DTT, 30 μM [^{14}C] GS-7340, 0.1% NP40 and varying amounts of enzyme in a final volume of 60 μl. The reaction mixture was incubated at 37°C and at 10, 30 and 90 minutes, 17μl of the reaction mixture was spotted onto a DE-81 filter. The filter was washed with 25mM Tris, pH 7.5 100mM NaCl, dried at room temperature, placed in vials containing 5ml of scintillation fluid. [^{14}C]-Metabolite X present on the filters was determined using a scintillation counter (LS 6500, Beckman). Activity was expressed as pmoles Metabolite X produced / minute / volume enzyme sample. Ester Hydrolase Specific Activity was expressed as pmoles Metabolite X produced / minute / μg protein.

Non-Specific Esterase Assay

Non-specific ester hydrolase activity was monitored by monitoring the enzymatic cleavage of alpha napthyl acetate (ANA) (Mastropaolo, W and Yourno, J 1981). This substrate

has been used for both the measurement esterase enzyme activity and *in situ* staining of esterases in tissue samples (Yourno, J and Mastropaolo, W. 1981; Yourno, J *et al.* 1981; Yourno, J *et al.* 1986). The method described is a modification of the assay described by Mattes, PM and Mattes, WB, 1992). Varying amounts of peripheral blood mononuclear cell (PBMC) extracts column fractions or pools were incubated with ANA at 37°C for 20 min. The final reaction conditions were: 10 mM sodium phosphate, pH 6.5, 97 μM ANA and varying amounts of enzyme in a final volume of 150 μl. The reaction mixture was incubated at 37°C and at 20 minutes, and the reaction was stopped by the addition of 20μl of 10mM Blue salt RR in 10% sodium dodecyl sulfate (SDS). The alpha napthyl-Blue salt RR product was detected by reading absorbance at 405nm. Activity was expressed as pmoles product produced /minute/ volume enzyme sample.

Extraction of GS-7340 Ester Hydrolase from Human PBMCs

Fresh human PBMC were obtained from patients undergoing leukophoresis; cells were shipped in plasma and processed within 26 h of draw. PBMC cells were harvested by centrifugation at 1200 X g for 5minutes and washed three times by re-suspension in RBC lysis buffer (155 mM NH_4Cl, 1 mM EDTA, 10mM $KHCO_3$). Washed cells ($29x10^9$) were suspended in 150 ml of lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 20 mM $CaCl_2$, 1 mM DTT and 1% NP40) and incubated on ice for 20 minutes. The PBMC crude extract was centrifuged at 1000 X g for 30 min to remove unlysed cells and the supernatant at 100,000 X g for 1h. The 100,000 X g supernatant (PBMC Extract: P0) was harvested (165ml) and the pellets (1000 X g and 100,000 X g pellets) were resuspended in 10 mM Tris, pH 7.4, 150 mM NaCl, 20 mM $CaCl_2$, 1 mM DTT and assayed for GS-GS-7340 ester hydrolase activity. Assays showed that < 2% of the GS-GS-7340 Ester Hydrolase enzymatic activity was present in the pellets. The cell extract was snap frozen in liquid Nitrogen and stored at –70°C.

Anion Exchange Chromatography

The PBMC Extract (15 X 10^9 cells, 75 – 85ml) was diluted 1:10, (vol: vol) with 25mM Tris, pH 7.5, 10% glycerol, 1mM DTT (Q15 Buffer A) and loaded onto an anion exchange column (2.5cm X 8.0 cm, Source Q15 (Amersham Biosciences)), previously equilibrated with Q15 Buffer A. Bound protein was eluted with a linear NaCl gradient (30 column volumes (CV)) to 0.5M NaCl. Eluting protein was detected by monitoring Absorbance at 280nm. Fractions

(12.0 ml) were collected and assayed for both GS-7340 Ester Hydrolase and ANA Esterase activity. GS-7340 Ester Hydrolase activity eluted as a single major peak at 50 – 75 mM NaCl. Recovery of Total GS-7340 Ester Hydrolase activity in the eluted fractions was 50 – 65% of total activity loaded. Significant ANA Esterase activity (30-40% of total activity loaded) was detected in the column FT; however, ~ 30% eluted in two peaks at 70 – 100 mM NaCl. Fractions containing GS-7340 Ester Hydrolase activity (Q15 pool) were pooled, snap frozen in liquid nitrogen and stored at –70°C.

Hydrophobic Interaction (HIC) Chromatography

The Q15 pool was defrosted and diluted 1:1, (vol: vol) with 25mM Tris, pH 8.0, 0.5 M $(NH_4)_2SO_4$, 1mM DTT, 10% glycerol BS-HIC Buffer A). 1M $(NH_4)_2SO_4$ was added to yield a final concentration of 0.5M $(NH_4)_2SO_4$ in the sample. The sample (300ml / 10 X 10^9 cells) was loaded onto a Butyl Sepharose HIC column (5ml HiTrap, Amersham Biosciences) previously equilibrated with BS-HIC Buffer A. Bound protein was eluted with a linear gradient (15 CV) decreasing to with 25mM Tris, pH 8.0, 1mM DTT, 10% glycerol. Eluting protein was detected by monitoring Absorbance at 280nm. Fractions (4.0 ml) were collected and assayed for both GS-7340 Ester Hydrolase and ANA Esterase activity. GS-GS-7340 Ester Hydrolase activity eluted as a single major peak at 200 – 75 mM $(NH_4)_2SO_4$. Recovery of Total GS-7340 Ester Hydrolase activity in the eluted fractions was 50 – 65% of total activity loaded. Significant ANA Esterase activity (85% of total activity loaded) was detected in the column FT; however, ~ 10-15% eluted in a peak at 450 – 300 mM $(NH_4)_2SO_4$. Fractions containing GS-7340 Ester Hydrolase activity (BS-HIC pool) were pooled, snap frozen in liquid nitrogen and stored at – 70°C.

Hydroxyapatite (HAP) Chromatography

The BS-HIC pool (40 ml / 10 X 10^9 cells) was defrosted, concentrated to 2.0ml using a 10kDa molecular weight cutoff concentrator (20ml Vivaspin concentrator, Viva Science, Carlsbad, CA), and diluted to 20ml with 1mM sodium phosphate, pH 6.85, 10% glycerol, 1mM DTT (HAP Buffer A). The sample containing the GS-7340 Ester Hydrolase activity was loaded onto a HAP column (0.75 ml, 5mm X 20mm; ceramic hydroxyapatite, BioRad, Hercules, CA), previously equilibrated with HAP Buffer A. Bound protein was eluted with a 40 CV gradient to 500 mM sodium phosphate, pH 6.85, 10% glycerol, 1 mM DTT. Eluting protein was detected

by monitoring Absorbance at 280nm. Fractions (0.5 ml) were collected and assayed for GS-7340 Ester Hydrolase. GS-7340 Ester Hydrolase activity eluted as a single major peak at 70 -85 mM sodium phosphate. Recovery of Total GS-7340 Ester Hydrolase activity in the eluted fractions was 40 -45% of total activity loaded. Fractions containing GS-7340 Ester Hydrolase activity (HAP pool) were pooled, snap frozen in liquid nitrogen and stored at –70°C.

High Resolution Gel Filtration Chromatography

The BS-HIC pool (5ml / 1.25 X 10^9 cells) was defrosted, concentrated to 0.05ml using a 5kDa molecular weight cutoff concentrator (20ml Vivaspin concentrator, Viva Science, Carlsbad, CA), and loaded onto a high resolution Gel Filtration column (8mm X 300mm, KW 802.5; Shodex, Thomas Instrument Co., Oceanside, CA), previously equilibrated with 25mM Tris, pH 7.5, 150mM NaCl, 10% glycerol, 20mM CaCl2, 1mM DTT (KW 802.5 column buffer). Eluting protein was detected by monitoring Absorbance at 280nm. Fractions (0.5 ml) were collected and assayed for GS-7340 Ester Hydrolase. GS-7340 Ester Hydrolase activity eluted as a single major peak at in fractions corresponding to an apparent molecular weight of 70 – 100 kDa. Recovery of Total GS-7340 Ester Hydrolase activity in the eluted fractions was >75% of total activity loaded. Fractions containing GS-7340 Ester Hydrolase activity (KW 802.5 pool) were pooled, snap frozen in liquid nitrogen and stored at –70°C.

Summary of GS-7340 Ester Hydrolase Purification

The following table summarizes the purification of GS-7340 Ester Hydrolase achieved. Protein was measured by a Coomassie Blue stain colorometric assay (Bradford Protein Assay, BioRad, Hercules, CA). The Specific Activity (pmoles Metabolite X produced / minute / μg protein) of the partially purified GS-7340 Ester Hydrolase varied from 666 to 1500. This represents a 222 – 750 fold purification from the PBMC extracts. Overall Recovery of GS-7340 Ester Hydrolase from PBMC extracts was approximately 10%.

Table 1c: Purification Summary of GS-7340 Ester Hydrolase

Sample name	PBMC	Protein concentration (mg/ml)	Volume (ml)	Protein (mg)	Total Activity (pmol/min)	Specific Activity pmol/min/μg	% Recovery
P0 PBMC	30 X 10^9	5.0	200	1000	2.0 – 3.0 X 10^6	2.0 – 3.0	
Q15 Pool		0.116 – 0.167	300	35 - 50	1.0 – 1.5 X 10^6	20 -42	~50
BS-HIC Pool		0.02 – 0.035	100	2.0 – 3.5	0.5 – 0.75 X 10^6	142- 375	~50
HAP Pool		0.02 – 0.03	10	0.2 – 0.3	0.2 – 0.3 x 10^6	666 - 1500	~40
						% Total Recovery	~10

Biochemical Characterization of GS-7340 Ester Hydrolase

Determination of the Isoelectric point (pI) of GS-7340 Ester Hydrolase

The isoelectric point (pI) of a protein is defined as the pH at which the protein has no net ionic charge. Chromatofocusing is a chromatographic procedure in which a negatively charged protein is bound to a hydrophilic column with a net positive ionic charge. The protein is loaded at a pH 1 to 2 pH units higher that its estimated pI, and the bound protein is eluted by generating a decreasing pH gradient using a pH 3.0 to 4.0 buffer. The proteins will be eluted at a pH corresponding to pI.

An aliquot of the BS HIC pool (20 ml, 5 X 10^9 cells) was concentrated to 4.0 ml and prepared for chromatofocusing chromatography by exchanging buffer using a desalting column. 1.0 ml aliquots of the concentrated BS HIC pool were loaded onto a 5.0 ml desalting column (5.0 ml HiTrap, Amersham Biosciences, Piscataway, NJ) previously equilibrated with 25mM ethanolamine, pH 7.8 (pH'd with iminodiacetic acid), 10% glycerol (Mono P Buffer A). The desalted GS-7340 Ester Hydrolase activity was loaded onto a chromatofocusing column (5mm X 5mm HR Mono P, Amersham Biosciences, Piscataway, NJ) previously equilibrated with Mono P Buffer A. Bound protein was eluted with a 20CV gradient to pH 3.6 with 10 ml / 100 ml

Polybuffer 74 (Amersham Biosciences) pH'd to 4.0 with iminodiacetic acid. This chromatofocusing protocol produces a linear pH gradient from pH 7.8 to pH 3.6. Eluting protein was detected by monitoring Absorbance at 280nm. Fractions (0.5 ml) were collected and assayed for GS-7340 Ester Hydrolase. GS-7340 Ester Hydrolase activity eluted as a single major peak at pH 5.5 to 4.5. Recovery of Total GS-7340 Ester Hydrolase activity in the eluted fractions was 65 -70% of total activity loaded. Fractions containing GS-7340 Ester Hydrolase activity (KW 802.5 pool) were pooled, snap frozen in liquid nitrogen and stored at –70°C.

Inhibition of GS-7340 Ester Hydrolases by Serine Hydrolase Inhibitors

Fluorophosphonate / fluorophosphate (Diisopropylfluorophosphate (DFP)) derivatives, isocoumarins such as 3,4 dichloroisocoumarin (3,4-DCI) and peptide carboxyl esters of chloro- and fluoro-methyl ketones (AlaAlaProAla-CMK, AlaAlaProVal-CMK, PheAla-FMK) are known effective inhibitors of serine hydrolases (Powers and Harper 1986; Delbaere and Brayer, 1985; Bullock *et al.* 1996; Yongsheng *et al.* 1999; Kam *et al.* 1993). Inhibition of the enzymatic production of metabolite X from GS-7340 was monitored using the following Ester Hydrolase Inhibition assay: Varying amounts of partially purified GS-7340 Ester Hydrolase and control enzymes (human leukocyte elastase (huLE), porcine liver carboxylesterase (PLCE)) were incubated with [^{14}C] GS-7340 in the presence and absence of varying amounts of known serine hydrolase inhibitors at 37°C for 10 – 90 min. The production of [^{14}C] Metabolite X was monitored by measuring the amount of radioactivity retained on an anion exchange resin (DE-81). The final reaction conditions were: 25 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.5, 100mM NaCl, 1 mM DTT, 30 μM [^{14}C] GS-7340, 0.1% NP40 varying amounts of enzyme and inhibitors (1.0μM – 1mM) in a final volume of 60 μl. The reaction mixture was incubated at 37°C and at 10, 30 and 90 minutes, 17μl of the reaction mixture was spotted onto a DE-81 filter. The filter was processed and the amount of [^{14}C]-Metabolite X present was determined as described above. Activity was expressed as pmoles Metabolite X produced / minute / volume enzyme sample. Inhibition of Ester Hydrolase and control hydrolases was expressed as percent activity present at a given concentration of inhibitor compared to hydrolase activity in the absence of the inhibitor. The results of the inhibition experiments are shown in Table 2A/B. The serine hydrolase inhibitors, 3,4-DCI and DFP inhibit GS-7340 Ester Hydrolase

with estimated IC50's of 4.0 and 30 μM, respectively. The peptide chloro- and fluoro-methyl ketones are less effective inhibitors with estimated IC50's of 100 –400 μM (Table 2 A / B).

Table 2A: Inhibition of GS-7340 Ester Hydrolase and Control Enzymes by Serine Hydrolase Inhibitors

	IC50 (μM)		
Inhibitor	**GS-7340 Ester Hydrolase**	**PLCE**	**huLE**
3,4-dichloroisocoumarin	4.0	250	3.0
MeOSuC-Ala-Ala-Pro-Ala-CMK	200-400	>1000	60
MeOSuc-Ala-Ala-Pro-Val-CMK	100	>1000	4.0
Biotin-Phe-Ala-FMK	100	>1000	100
DFP	30	0.05	-

Table 2B: Inhibition of GS-7340 Ester Hydrolase and Control Enzymes by Serine Hydrolase Inhibitors

	Inhibitor (μM)	**Relative Activity (%)**	**IC50 (μM)**
GS-7340 Ester Hydrolase			
3,4-dichloroisocoumarin	1.0	100	4.0
	10	25	
	100	5	
	1000	<2	
DFP	1.0	100	30-40
	10	90	
	100	35	
	1000	<2	
Biotin-Phe-Ala-FMK	1.0	100	100
	10	95	
	100	50	
	1000	<2	
PLCE			
3,4-dichloroisocoumarin	1.0	100	250
	10	100	
	100	90	
	1000	20	

DFP	0.001	100	0.05
	0.01	90	
	0.1	20	
	1.0	<2	
Biotin-Phe-Ala-FMK	1.0	100	>1000
	10	100	
	100	100	
	1000	80	
huLE			
3,4-dichloroisocoumarin	1.0	100	4.0
	10	25	
	100	5	
	1000	<2	
Biotin-Phe-Ala-FMK	1.0	100	100
	10	93	
	100	48	
	1000	<2	

Summary of Biochemical Characterization of GS-7340 Ester Hydrolase

Summarizing,GS-7340 Ester Hydrolase is a novel enzyme characterized by being capable of being recovered from human PBMCs by a process comprising

(a) lysing human PBMCs;

(b) extracting the lysed cells with detergent;

(c) separating the solids from supernatant and recovering the supernatant;

(d) contacting the supernatant with an anion exchange medium;

(e) eluting the Hydrolase from the anion exchange medium;

(f) contacting the eluate with a hydrophobic chromatographic medium; and

(g) eluting the Hydrolase from the hydrophobic chromatographic medium.

GS-7340 Ester Hydrolase is useful in screening candidate compounds to assess the likelihood that they can be processed to form depot metabolites in lymphoid tissue. The candidates are assayed in the same fashion as described herein for GS-7340, taking into account differences in the nature of the suspected substrate as will be apparent to the ordinary artisan.

GS-7340 Ester Hydrolase optionally is labelled with a detectable group such as a radiolabel or covalently bound to an insoluble matrix such as Sepharose using techniques heretofore employed for other enzymes having similar properties, as will be apparent to the ordinary artisan.

GS-7340 Ester Hydrolase has the following properties:

1) GS-7340 Ester Hydrolase can be partially purified from fresh PBMC Extracts: SA = 666 -1500 pmoles MetX/min/ug protein.

2) GS-7340 Ester Hydrolase can be separated from non-specific Esterases capable of cleaving alpha-naphthyl acetate (ANA), a non-specific substrate shown to be cleaved by many carboxylesterases and hydrolases.

3) Multiple GS-7340 Ester Hydrolase activity peaks are not eluted from columns during purification.

4) The MW of GS-7340 Ester Hydrolase on Gel Filtration is ~ 70 - 100kDa

5) The pI of GS-7340 Ester Hydrolase is pH 4.5 -5.5

6) Evidence to date suggests that the SA of isolated GS-7340 Ester Hydrolase is likely to be > 10,000.

7) The serine hydrolase inhibitors, 3,4-DCI and DFP inhibit GS-7340 Ester Hydrolase with estimated IC50's of 4.0 and 30 μM, respectively. The peptide chloro- and fluoro-methyl ketones are less effective inhibitors with estimated IC50's of 100 –400 μM (Table 2 A / B).

References:

Bullock, TL *et al.* 1996 *J Mol Biol* 255: 714-725.

Delbaere, LT and Brayer, GD 1985 *J Mol Biol* 183:89-103

Kam C *et al.* 1993 *Bioconjugate Chem* 4: 560-567

Mastropaolo, W and Yourno, J 1981 *Analytical Chemistry* 115: 188-193

Mattes, PM, and Mattes, WB, 1992. *Toxicol. Appl. Pharmacol.* 114:71-76

McGuigan, CPW *et al.* 1998a *Antiviral Chem and Chemotherapy* 9: 109-115

McGuigan, CPW *et al.* 1998b *Antiviral Chem and Chemotherapy* 9: 473-479

Powers, JC and Harper, JW 1986 Inhibitors of serine proteinases. In Proteinase Inhibitors (AJ Barrett and G Salvesen, Eds.) Elsevier, Amsterdam, New York, Oxford, pp55-152)

Saboulard, DL *et al.* 1999 *Molec Pharmacol* 56:693-704

Siddiqui, AQC and McGuigan, CPW 1999 *J Med Chem* 42:4122-4128

Valette, GA *et al.* 1996 *J Med Chem* 39:1981-1990

Yongsheng, the linker *et al.* 1999 *Proc Natl Acad Sci* 96:14694-14699

Yourno, J and Mastropaolo, W. 1981 *Blood*, 58:939-945

Yourno , J *et al.* 1981. *Blood*, 60: 24-29

Yourno, J *et al.* 1986 *J Histochem and Cytochem* 34:727-33)

Example: Candidate Compounds

A large number of examples describing the preparation of candidate compounds active against HIV protease, HIV integrase and HIV polymerase (non-nucleotide reverse transcriptase inhibitors, or NNRTIs) are found in copending applications and are set forth below. These compounds are examples of candidate compounds that are typical of those which are suitable for use in the method and libraries of this invention.

Incorporation by Reference

All publications and patent applications cited herein are incorporated by reference to the same extent as if the full text of each individual publication or patent application was contained herein. The incorporated text will be apparent from context if not specifically set forth.